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THE OXYTOCIN SECRETION AND MILK LETDOWN DURING MILKING IMMEDIATELY AFTER THE CHANGE OF MILKING AND HOUSING CONDITIONS

SEKRÉCIA OXYTOCÍNU A EJEKCIA MLIEKA POČAS DOJENIA BEZPROSTREDNE PO ZMENE PODMIENOK DOJENIA A USTAJNENIA

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ABSTRACT: The effect of relocation to known surroundings immediately before machine milking on the oxytocin (OT) secretion and milk yield was studied. Twelve Brown Swiss cows in their fifth week of lactation were used. Animals were relocated immediately before the evening milking within the same stable from loose housing and milking in the parlour to tie stalls where they were milked. All cows had previous experience with this type of housing. Milk yields were recorded two evenings before relocation (control) and during the first two consecutive evening milkings after relocation (first and second milking). Blood was collected during the first and second evening milking after relocation. Relocation of cows significantly reduced milk yield, and this reduction was significantly recovered during the second evening milking. The OT concentrations significantly increased during the second milking compared with the first milking after relocation. Positive correlation between increased OT concentration and increased milk yield from first to second evening milking ($r = 0.532$) was calculated. In conclusion, the lower milk yield after the change of housing and milking conditions can be concomitant with disturbed OT release but a mechanism responsible for decreased milk yield is unclear.

dairy cow; milking; relocation; oxytocin; milk yield

ABSTRAKT: Zisťovali sme vplyv presunu kráv do známeho prostredia bezprostredne pred dojením na sekréciu oxytocínu (OT) a úžitkovosť. Do pokusu bolo zaradených 12 dojníc hnedého švajčiarskeho plemena, ktoré boli v piatom týždni laktácie. Okamžite pred večerným dojením boli dojnice presunuté zo systému voľného ustajnenia a dojenia v dojárni do systému ustajnenia s priväzovaním a dojením na stojisku. Obidva systémy sa nachádzali v tom istom objekte. Zvieratá mali predchádzajúcu skúsenosť s týmto ustajnením. Charakteristiky dojenia sme zisťovali počas štyroch večerných dojení – dva dni pred presunom (kontrolné) a dva dni po presune (prvé a druhé dojenie). Odber krvi sme robili len počas dvoch večerných dojení po presune. Presun kráv do známych podmienok preukazne znížil množstvo nadojeného mlieka s opätovným signifikantným zvýšením počas nasledujúceho večerného dojenia. Počas druhého dojenia v porovnaní s prvým dojením po presune došlo k preukaznému zvýšeniu hladiny OT. Medzi nárastom hladín OT a množstvom mlieka z prvého na druhé večerné dojenie po presune sme vypočítali pozitívnu koreláciu ($r = 0,532$). Zníženie nádoja po presune bolo sprevádzané poruchami sekrécie OT, avšak mechanizmus zodpovedný za toto zníženie zostáva nejasný.

dojnice; dojenie; presun; oxytocín; úžitkovosť

INTRODUCTION

The ejection of alveolar milk, evoked by oxytocin (OT) release from the pituitary gland into the blood in response to tactile teat stimulation, is essential for fast and complete milk removal in dairy cows (Gorewit et al., 1992; Mayer et al., 1991; Bruckmaier et al., 1993). Contraction of myoepithelial cells occurs when OT is reaching the threshold level, which is about 3–5 ng/l (Schams et al., 1984). For complete milk removal the continuously elevated OT release during the entire milking is required as well (Bruckmaier et al., 1994)

Milk removal can be disturbed under various conditions at the central (Goodman and Grosvenor, 1983; Bruckmaier and Blum, 1998; Tančin et al., 1993, 1995) and peripheral levels (Bruckmaier et al., 1991; Blum et al., 1989). Central inhibition involves the cessation of the OT release from the posterior pituitary gland in response to the tactile stimulation and it is well described during milking after relocation to unknown and unfamiliar surroundings. Peripheral inhibition represents the lack of OT effect at the level of the udder. It means that milk removal can be negatively influenced even when OT release occurred. However, relocation of cows

to new housing can negatively influence milk yield during first milking (Varner et al., 1983; Brestenský et al., 1988) without the change of milk ejection. Also the stereotype of routine during milking can support milk removal and milk flow (Velitok, 1977). However, under the last mentioned conditions OT was not measured.

In this experiment we have therefore focused on the OT release and milk yields during the adaptation of cows after relocation within the same stable from loose to tie housing immediately before milking.

MATERIAL AND METHODS

Animals and experimental procedures

Six primiparous and six multiparous Brown Swiss cows on their fifth week of lactation were used. The cows belong to the Institute of Physiology TU München Freising-Weihenstephan. They had free access to a mixed ration and received additional concentrates in relation to their milk production.

Pre-experimental period: All cows had previous experience with tie housing, milking in the stall and frequent blood collection. These animals were housed, milked and bled during the first 6 days after parturition here. Thus relocation could be considered as relocation to the known surroundings.

Experiment: Cows were relocated immediately before evening milking within the same stable from loose to tie housing on 35th day post partum. Thus animals in tie housing were separated from the herd, but they could see and hear the herd. Two consecutive evening milkings before relocation were considered as control for milk yield. Milk yield was also measured and blood collected during two consecutive evening milkings after relocation (first and second evening milking). Cows were milked twice daily at 7.00 and 17.30 hours – before relocation in milking parlour and after relocation in stall using a bucket milking installation with an integrated mobile milk flow recording device (Bruckmaier and Blum, 1996).

Blood collection

Blood samples of 10 ml were collected via jugular catheter into tubes containing 200 µl of a solution with EDTA (300 µmol/l, Merck, Darmstadt, Germany) plus acetylsalicylate (1%, Serva, Heidelberg, Germany), cooled in an ice bath, centrifuged at 3 000 g, aliquoted and stored at –20 °C until assayed. Jugular catheter was inserted within 1 hour after morning milking on day of relocation (35th day). Blood samples were taken at –5 and –1 min before, and 0, 0,5; 1; 1,5; 2; 2; 3; 4; and 5 after the start of 1 min hand stimulation and machine milking.

Oxytocin determination

OT was determined radioimmunologically as originally described for cattle (Schams et al., 1979) after the extraction with SEP-PAK C18 cartridges (Water Assoc., Inc., USA). The antiserum showed no cross-reaction with related peptides such as lysine- or arginine-vasopressin or anterior pituitary hormones. The extraction recovery was at 0.8, 1.6, 3.2 and 6.4 ng/l on average 76 ± 10%, (n = 6 assays). The within-assay coefficient of variation (C V) varied from 5.9 to 7.8% and the between-assay C V from 11.2 to 16.9 % in samples with high (17.2 ± 1.9 ng/l) and low (1.6 ± 0.3 ng/l) OT concentration.

Statistical analysis

Data are presented as a mean ± s.e.m. The area below the curve AUC/min (AUC) for each individual cow was calculated from OT concentrations during the first 5 min after the start of stimulation and milk removal. The effect of cow and treatment was tested by two-way ANOVA (Microsoft Excel). AUC and milk characteristic data were used to calculate differences between treatments by pair t-test (Microsoft Excel). Differences were assumed as significant if $P < 0.05$.

RESULTS

Milk yields and OT secretions during milking in primiparous and multiparous cows in response to relocation are documented in Tab. I. There were significantly higher milk yields in multiparous than primiparous cows but primiparous cows showed the tendencies to have a nonsignificantly higher OT secretion ($P < 0.1$). Milk yield after relocation of all cows was significantly reduced and this reduction was significantly recovered on the second evening milking (Tab. I). The OT concentrations significantly increased during the second evening milking compared with the first milking after relocation (Tab. I).

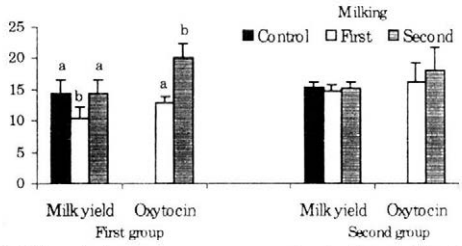
We have calculated a positive correlation between increased OT values and milk yield from first to second evening milking (r = 0.532). The cow was a significant factor. From all individual increases of milk yield from first to second evening milking we have created two

I. Effect of relocation from loose to tie housing on milk yield and oxytocin secretion

		Milk yield (kg)			Oxytocin (ng/l)	
		control	first	second	first	second
Primi	$\bar{x}$	11.3^{Aa}	9.2^{Ba}	10.9^{Aa}	23.9^{A}	30.2^{B}
	s.e.m.	0.9	1.2	0.8	6.2	5.9
Multi	$\bar{x}$	17.2^{b}	15.1^{b}	17.3^{b}	13.3^{A}	18.1^{B}
	s.e.m.	0.7	0.7	0.7	2.2	4.5
Total	$\bar{x}$	14.2^{A}	12.1^{B}	14.1^{A}	18.6^{A}	24.2^{B}
	s.e.m.	0.8	0.9	0.9	2.2	2.7

Different superscripts within a treatment (A, B) and between parity (a, b) indicate significant differences $P < 0.05$

Control = two evenings milking before relocation in the parlour; first = first evening milking after relocation; second = second evening milking after relocation in the stall, resp.



1 Effect of relocation on oxytocin secretion (ng/l) and milk yield (kg) in two selected groups

Different superscripts in milk yield or oxytocin indicate significant differences between milkings $P < 0.05$
Control, first and second see in Tab. I

groups. The selection criterion for group I was like this: the first five animals with greatest increase of milk yield and for group II: the first five animals with lowest values. There was no effect of parity. As it is shown in Fig. 1, animals characterised by the negative effect of relocation on milk yield (group I) showed a significant increase of OT release from first to second milking as compared with group II. Also in group I the peak milk flow and time of milking were increased from first to second milking (2.8 ± 0.9 kg/min vs 3.2 ± 0.3 kg/min, $P < 0.05$, 6.3 ± 0.9 min vs 6.8 ± 0.8 min, respectively). In the second group peak milk flow and time of milking did not change from first to second milking (3.4 ± 0.5 kg/min vs 3.2 ± 0.5 kg/min, 6.0 ± 0.7 min vs 6.1 ± 0.5 min, respectively).

DISCUSSION

Though we do not have the oxytocin data from control milking we assume that it is also possible to show the effect of relocation on OT secretion by comparing data obtained during milking immediately after relocation (first evening milking) and second evening milking. There is evidence about a faster adaptation to unknown and unfamiliar surroundings observed in milk yield than in OT concentration (Bruckmaier et al., 1996). On the basis of this information we could observe a higher suppression of OT release from control to first milking after relocation than it was the increase of OT release from first to second evening milking after relocations. Regardless of this speculation we could clearly demonstrate that relocation even to known surroundings negatively affects OT secretion and milk yield as well. This effect is further supported by the individual profiles of OT secretion divided into two groups. Animals with significantly increased milk yield from first to second evening milking (first group) also significantly increased OT secretion. Animals in the second group showed a small increase of milk yield that was attended by no changes in OT secretion.

Emotional stress (relocation, social isolation) stimulates the cortisol and endogenous opioid beta-endorphin release (Varner et al., 1983; Bruckmaier et al., 1993; Hashizume et al., 1994). There are results concerning the rat which show that under the stress conditions the opioid system can negatively influence OT release on the central levels in response to stimulus (Bicknell and Leng, 1982; Russell et al., 1993) but not in dairy cows (Wellnitz et al., 1997). On the other hand, the opioid system in dairy cows can influence OT release during milking under normal conditions (Schams et al., 1998). Another mechanism, considered to be involved in the OT and milk ejection regulation, is a noradrenergic system (Blum et al., 1989; Lefcourt and Akers, 1991; Lefcourt et al., 1997). However, phentolamin (α-adrenergic blocker) could not restore OT release during milking under stress conditions (Wellnitz et al., 1997) though α-adrenergic receptor stimulation can suppress the milk flow (Bruckmaier et al., 1991).

Decreased OT concentration during first milking connected with lower milk yield after relocation in the first group of animals could indicate the suppressive effect of relocation on the OT secretion on the central level. However, on the basis of threshold principle of OT effect on milk ejection, the milk yield in our experiment could be negatively influenced mainly at the level of the udder and not by lower OT concentration. The sympathetic nervous system plays an important role in regulation of the efficiency of the milk ejection reflex. Probably in the cows of group I the activation of adrenergic receptor in the udder tissue could reduce the free flow of milk from storage place during milking as it is also documented by others (Blum et al., 1989; Bruckmaier et al., 1991). The tissues of the large mammary ducts above the gland cistern and of the mammary parenchyma contain α-adrenergic receptor (Hammon et al., 1994).

Thus the inhibition of OT release and milk yield in some cows can be ascribed to stress from relocation. If endogenous opioids or other mechanisms are involved in OT secretion during stress reaction, we assume that two different reactions of cows to relocation to a known place could result in individual sensitivity of animals to stress from the change of surroundings. These individual differences are observed at the level of the hypothalamic-pituitary-adrenal axis (Borell and Ladewig, 1992). There is also evidence that cows that best recognised aversive people increased the amount of residual milk (Rushen et al., 1999) supporting individual sensitivity to stressor, but oxytocin was not measured.

In conclusion the relocation to known surroundings can negatively influence milk yield but the lowered OT release is not a reason. Probably individual sensitivity of animals to the stress could result in OT release during milking.

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IMMUNOCYTOCHEMISTRY OF HEAT SHOCK PROTEINS HSP70 IN PIG LIVER AFTER A PARASITIC INVASION*

IMUNOCYTOCHEMICKÁ DETEKCE STRESOVÝCH PROTEINŮ HSP70 V JÁTRECH PRASAT PO PARAZITÁRNÍ INVAZI

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ABSTRACT: The presence of heat shock proteins 70 (HSP70) in pig liver tissue was detected using the immunocytochemical method as a response to the stress represented by parasitic invasion (*Ascaridae*). An increased occurrence of HSP70 was detected in the cytoplasm of hepatocytes in the close vicinity of the *vena centralis*. After using the antibody anti-HSP70 a positive reaction was detected in the nuclei of the hepatocytes in certain areas of the liver. A strong reaction was observed in the cytoplasm of cells forming necrotic foci. Multiplied connective tissue was plentifully infiltrated by eosinophilic granulocytes, which also demonstrated positivity. A slight positive reaction was also present in the endothelial cells of blood vessels as well as in the multiplied Kupffer cells.

heat shock proteins; HSP70; liver cell; eosinophils; immunocytochemistry

ABSTRAKT: Imunocytochemicky byla detekována přítomnost proteinů tepelného šoku z rodiny HSP70 v jaterní tkáni prasat v odpovědi na stres, jakým je parazitární napadení škrkavkami. Zvýšený výskyt HSP70 byl detekován v cytoplazmě hepatocytů v těsném sousedství *vena centralis*. Po použití protilátky anti-HSP70 byla detekována pozitivní reakce v jádrech hepatocytů v určitých okrscích jater. Silná reakce byla pozorována v cytoplazmě buněk vytvářejících nekrotická ložiska. Zmnožená vazivová tkáň byla hojně infiltrována eozinofilními granulocyty, které jevily rovněž pozitivitu. Slabá pozitivní reakce byla přítomna také v endotelových buňkách krevních cév i ve zmnožených Kupfferových buňkách.

stresové proteiny; HSP70; jaterní buňka; eozinofilní granulocyty; imunocytochemická reakce

INTRODUCTION

Several types of simple as well as highly specialised cells respond to stimuli causing disorder in their structure and/or function by the induction of specific proteins called stress proteins in the literature. Heat stress has been revealed to be the primary factor causing their expression in the cell and, for this reason, the term heat shock proteins abbreviated to HSPs, is ordinarily used (Lindquist and Craig, 1988; Welch, 1992; Morimoto et al., 1994). These proteins are identified by a numerical index corresponding to their molecular weight (Subjeck and Shyy, 1986).

We concentrated our attention on one set of stress proteins described as the HSP70 family. The HSP70, a set of universally conserved proteins, comprises a whole range of individual proteins, which act as molecular chaperones. Each of them bind ATP *in vitro* (Becker and Craig, 1994) and their expression is evoked by a large range of stressors. When cells are exposed to stress, the cellular homeostasis is damaged initiating the transcription of a part of the DNA molecule. This transcription triggers increased synthesis of HSPs (Morimoto et al., 1994). These stress proteins are involved in the accumulation and reparation of damaged protein structures. They mark the denatured proteins and some of them even eliminate damaged proteins (Hightower, 1980; Welch, 1992).

An invasion of helminths in the liver greatly taxes the organism and, protective mechanisms are therefore called forth. As previously said, the synthesis of stress proteins is one of the intracellular protective mechanisms. At the present time, there is intensive research into stress proteins and, inter alia, it has been determined that one type of stress need not have any influence on the size of response to another type (Blake et al., 1991a, b, c).

The stressors, which affect the liver cells, cause changes in the cells at the ultrastructural level. Different cellular compartments are stricken including the plasma membrane, the mitochondria, the endoplasmic retriculum, the cytoskeleton, ribosomes and the nucleus/nucleolus. Cellular defence reaction manifests itself through synthesis and transport of the newly synthesised proteins into damaged organelles and into the nucleus. Most of the stress conditions endanger the structural integrity

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of the proteins. The binding of HSP70 to the damaged proteins stabilises them and inhibits their further degradation (Gething and Sambrook, 1992; Becker and Craig, 1994).

The strong invasion of helminths into the liver causes remarkable pathomorphological changes in this organ. We can observe haemorrhages, parenchyma and fat dystrophy, and focal necrosis. During reparation of the liver tissue, damaged by migration of the parasites, many processes arise: focal fibrosis with remarkable infiltration by eosinophilic granulocytes, and simultaneously a thickening and blurring of the liver sheath. These phenomena are known as milk spots on the liver (Popper, 1982).

The aim of this study was to verify whether liver cells react to parasitic stress by synthesis of stress proteins. For their detection and precise localisation within the tissue or within the cells we applied the monoclonal antibody anti-HSP70 (or a monoclonal antibody against HSP70) with using the immunocytochemical reaction in histological sections of affected liver tissue.

The current investigation was undertaken to observe the impact of stress proteins (HSPs) on the liver parenchyma following an invasion of parasites.

MATERIAL AND METHODS

Animals

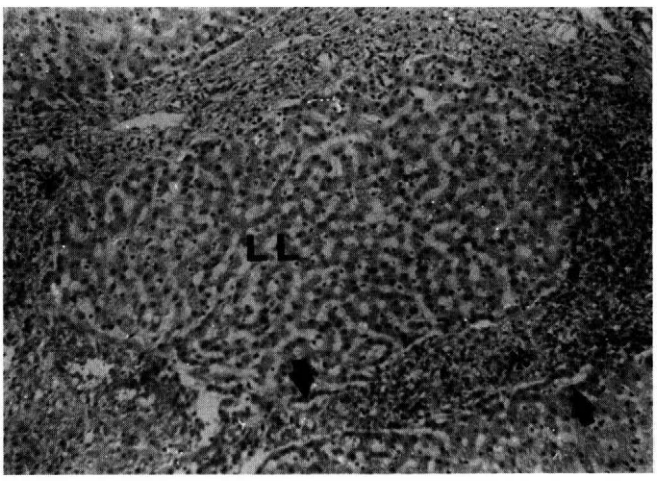
The biological experimental material was collected during veterinary inspection from about 30 hybrid pigs (combination of crossing /BU x L/ x ÈVM) in the slaughterhouse of the Masokombinát a.s. firm in Praha-Písnice. Small samples of liver were obtained from organs and their parts that showed visible changes, typical for parasitic invasion. The control ones were obtained from animals without any visible or histological changes (tested later).

Histological method and staining

Tissue blocs (1 x 1 cm) were fixed with 4% formaldehyde for 1–2 hours, and were then processed by a routine histological technique and embedded in paraffin. Two series of seven-micrometer paraffin sections were made. The first part of sections from the first series was stained with routine hematoxylin-eosin staining and the second part was stained using a 1% aqueous solution of toluidine blue and safranine (Martin-Partido et al., 1986).

Immunocytochemical detection of HSP70

The second series of paraffin sections was stained with immunohistochemical method to demonstrate stress proteins using mouse monoclonal antibody against HSP70 (anti-HSP70). After deparaffinization the sections were pre-treated with hydrogen peroxidase (0.3% solution in methanol for 30 minutes) to suppress endogenous peroxidase activity. Non-specific bindings were blocked using a nonimmune horse serum. The slides with sections were then covered by the specific monoclonal antibody anti-HSP70 (StressGen, Biotechnologies Corp. Canada) in a dilution of 1 : 100 and 1 : 200 in a humidified chamber at 37 °C for 30 minutes. Another application in a dilution of 1 : 500, overnight, at 4 °C, proved to be the best. The anti-mouse biotinylated secondary antibody (37 °C for 30 minutes) was followed by a peroxidase-labelled avidin-biotinylated horseradish peroxidase complex and was applied for 45 minutes. The colour was developed using diaminobenzidine as the chromogen, a Vecta stain ABC kit (Vector Laboratories, Canada) was used. The sections remained without counterstaining. For every sample a negative control was made i.e. the whole process without application of a specific antibody.



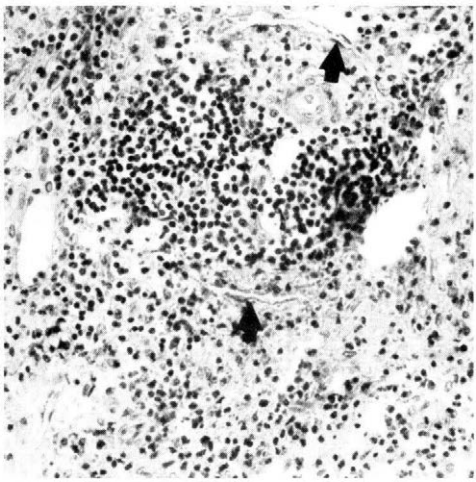
1. A part of liver stained with hematoxilin-eosin, LL-liver lobule, asterisk-connective tissue richly infiltrated by eosinophilic granulocytes, arrows-dilated sinusoids; 1 500x

During the final step the sections were dehydrated in alcohol, mounted in a mounting medium, then examined and photographed with a light microscope JenaMed II (Zeis, Jena).

RESULTS

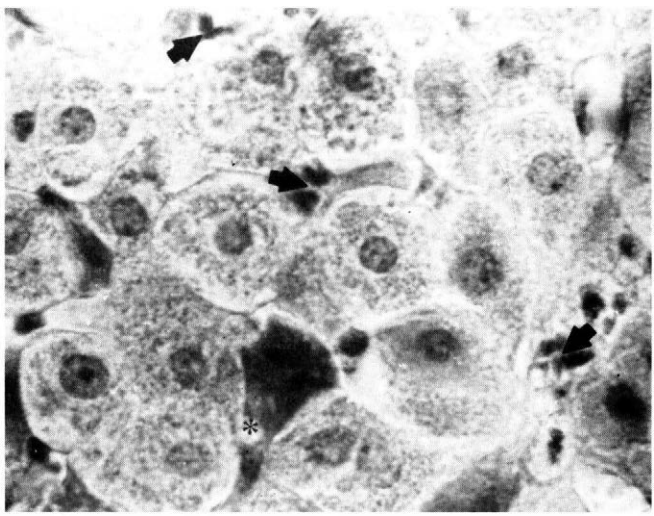
Histology and histopathological evaluation of liver tissue samples

When examining liver from various animals we can see a very uniform image – haemorrhages, parenchyma, and fat dystrophy, and numerous necrotic foci in damaged parts of the liver. Following the healing of the consequences of parasitic invasion, we find focal fibrosis with strong eosinophilic infiltration in the damaged liver tissue (Fig. 1). This amplification of the connective tissue is very conspicuous in comparison with control samples, and, parallel with its development, there is a decrease in liver parenchyma. The structural scheme of the liver lobule is disrupted, the portal spaces and their structures (artery, veins and bile duct) are disintegrated (Fig. 2), and in certain places obliteration of the bile duct is visible. In many places we find a great number of lymphocytes, and lymphocyte infiltration. Liver sinusoids are strongly dilated (Figs. 1, 2). The liver parenchyma is arranged in structural units called classical liver lobules and each liver lobule consists of rows of radially disposed hepatocytes. In many places, we can demonstrate necrotic changes in the hepatocytes. Strong vacuolisation of the cells and a growing density of nuclear material (pycnosis of nuclei) accompany these changes.

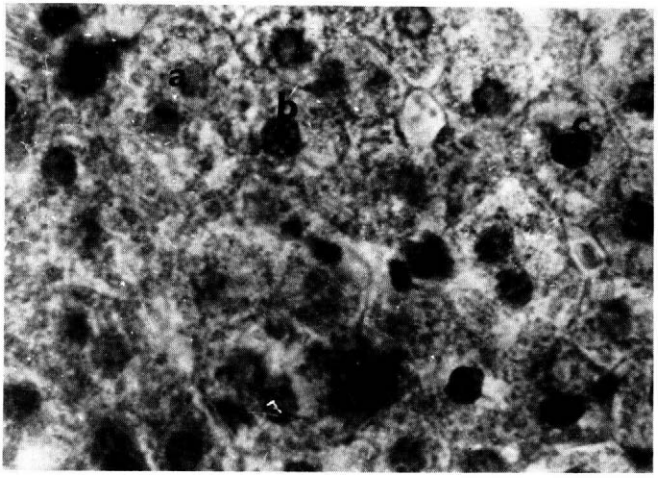


2. A part of disintegrated portal space with eosinophil infiltration, arrows-dilated sinusoids, hematoxilin-eosin staining; 1 500x

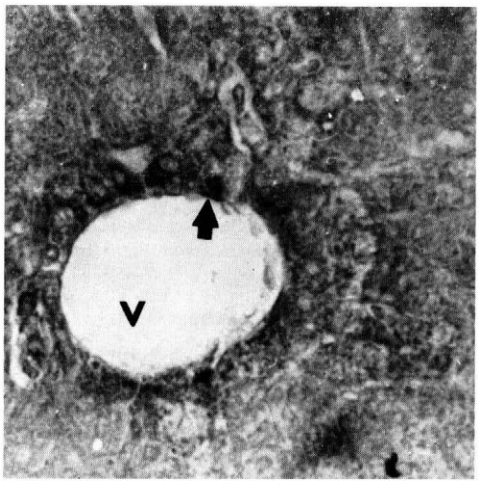
In the sections stained with hematoxylin-eosin, clumps of these damaged cells show conspicuous eosinophilia. Cell death can be represented by two mechanisms: necrosis or apoptosis. Necrosis is accompanied by many morphological changes. Intracellular organelles, most notably the mitochondria, and the entire cell, swell and rupture (cell lysis). The chromatin pattern is slowly changed. After breakdown of the plasma membrane, the cytoplasmic contents, including lysosomes with lysosomal enzymes, are released into the extracellular fluid. Apoptosis is a mode of cell death that occurs under normal conditions. It is called physiological cell death and it is characterised by chromatin aggregation, nuclear and cytoplasmic condensation (Fig. 3), and partition of the cytoplasm and nucleus into membrane bound-vesicles – apoptotic bodies (Fig. 3). In order to recognise the apoptosis in our material, we observed the occurrence of apoptotic bodies that are deposited intracellularly or extracellularly, the vacuolisation of cytoplasm and pycnosis of nuclei (Fig. 4). We identi-



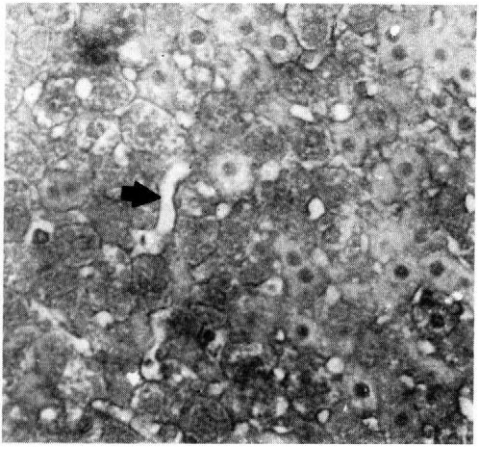
3. Apoptotic hepatocyte shows nuclear and cytoplasmic condensation (asterisk), presence of apoptotic bodies is marked by arrows. Toluidine blue and safranine staining; 5 000x



4. Apoptotic hepatocytes with vacuolisation of cytoplasm (a, b, c), arrow-pycnosis of cell nucleus. Toluidine blue and safranine staining; 5 000x



5. Detection of HSP70; positive reaction in centrilobular hepatocytes which rim the central vein (V), arrow-positivity in endothelial cell; 2 500x



6. Detection of HSP70; positive reaction in nuclei of hepatocytes, arrow-a dilated sinusoid; 200x

fied the apoptotic cells by means of toluidine blue and safranine staining.

Detection of HSP70

The expression of HSP70 in liver tissue after parasitic invasion was observed immunocytochemically using the antibody anti-HSP70. The centrilobule hepatocytes that rim the central vein very often showed a strong positive staining for HSP70 (Fig. 5). This reaction was much stronger in comparison with those in neighbouring hepatocytes. Cytoplasm, in particular, reacted strongly to the anti-HSP antibody. In contrast, the nuclei in these hepatocytes were stained weakly. Hepatocytes localised in marginal (peripheral) regions of the liver lobule or hepatocytes in the close vicinity of the portal space possessed nuclei demonstrating a stronger positive reaction (Fig. 6).

Some dispersed hepatocytes with a large quantity of vacuoles in the cytoplasm and with signs of cellular injury responded with strong immunostaining with anti-HSP70 (Fig. 7). But it seems that this reaction is not strictly bound to individual cells. A positive reaction has a tendency to pass from one cell to another and has a diffuse appearance.

Eosinophilic granulocytes profusely infiltrating the liver connective tissue also demonstrate HSP positivity that is localised in the granules even when this reaction is not very pronounced.

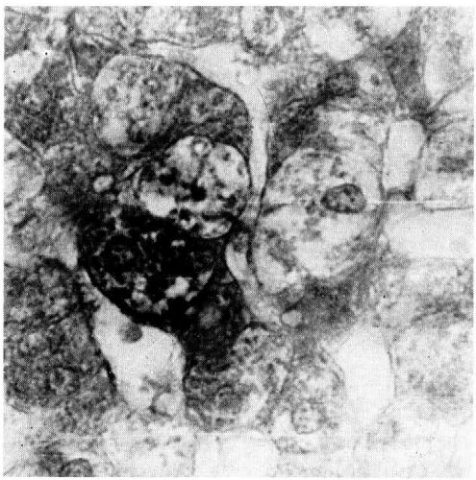
We found strong positive immunostaining of HSP70 in the endothelial cells of blood vessels, namely in the *vena centralis* (Fig. 5) as well as in the endothelial lining liver sinusoids, in small veins and also on the borderline between the media and the adventitia of arteries (Fig. 8) lying in the portal space.

Kupffer cells line the lumen of the hepatic sinusoids. They are usually stellate in shape and are mostly distributed in the sinusoids around the portal tract. Kupffer cells constitute about 85% of the fixed macrophages of the reticulo-endothelial system and they have morphologic features typical of macrophages. These cells also showed a positive reaction to HSP70 (Fig. 9).

Lymphocytes, which infiltrate the connective tissue of portal spaces as well as the epithelial cells of bile ducts, did not show any positive reaction with the antibody. Similarly, the smooth muscle cells of the portal veins and also the liver arteries showed no indication of HSP70.

A very weak positivity could be demonstrated in the negative controls processed without antibody. That fact could be due to the incomplete suppression of endogenous peroxidases by which we also explain the non-specific positivity of blood elements present in the tissues.

The control samples obtained from healthy animals did not show any pathological changes. These tissues express only a weak positive reaction in endothelial cells. Seldom incident hepatocytes with symptoms of apoptosis also reacted with the anti-HSP antibody.

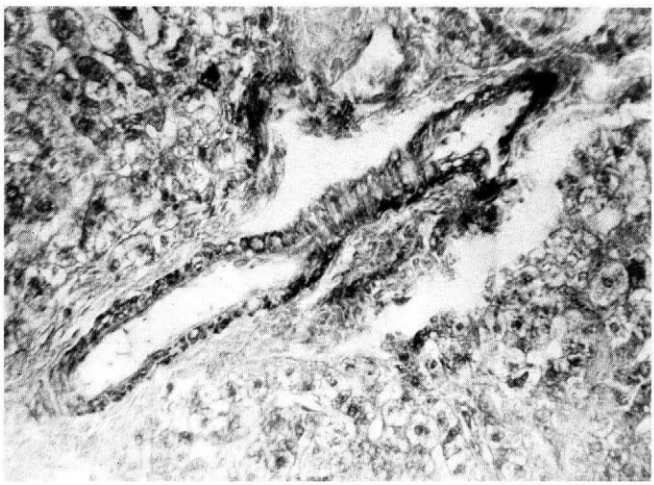


7. Detection of HSP70; strong positive reaction in the hepatocyte with vacuolated cytoplasm; 6 000x

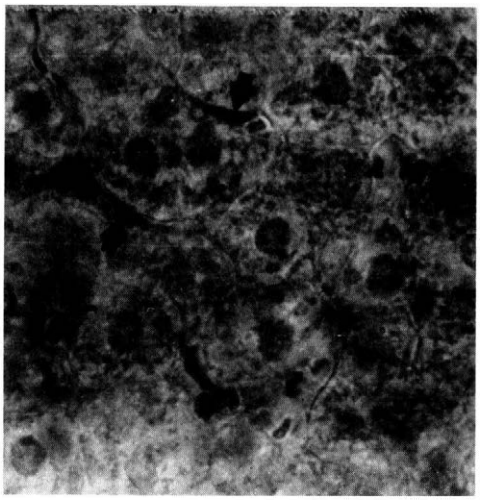
DISCUSSION

The fact that heat shock response by the way of HSPs synthesis is one form of the cellular adaptation mechanism to stress which injures membrane structures in the cell or the integrity and structure of proteins is common knowledge (Williams et al., 1993). From this it can be concluded that there are many stressors that induce synthesis of HSPs, probably using the same signal pathway. It is known from accessible literature that just such an expression of HSP70 is stimulated by a large spectrum of agents (Mestril and Dillmann, 1995). All agents operate via the sulfhydryl groups of aminoacids. Only functioning proteins or proteins in the process of maturation are attacked.

We realise that the definition and understanding of the term stress at cellular level is rather problematical because stress of the organism as a whole does not represent the stress on all or some of its cells. To a certain degree, cellular stress is influenced by the intensity or disruption of cell metabolism, by the stage reached in the cell cycle and so on, and this phenomenon manifests itself especially during exceptional disturbance.



8. Detection of HSP70; positivity in the wall of small artery; 2 000x



9. Detection of HSP70, arrows-positive reaction in Kupffer cells; 8 000x

This problem is therefore divided into two groups: one group is represented by exogenous physical factors, which induce stress, and the second group represents endogenous, metabolic and toxic factors.

The liver is an organ with many functions – absorption of nutrients, phagocytosis via the phagocytic cells, endocrine and exocrine activity, protein synthesis, bile secretion, storage of metabolite, detoxification, and hemopoesis – and for this reason it is a suitable and sensitive indicator of the functional state of the organism.

Heat or chemical stress is often accompanied by protein denaturation and HSPs are able to repair the conformation of denatured protein by binding to it (Chaloupka, 1994). This response is probably explained by the fact that the anti-HSP70 reaction within the normal hepatocytes was weak and without specific binding into certain cell structures. The reaction was stronger only in foci of hepatocytes that are thought to be apoptotic hepatocytes. These findings are in correlation with research data in literature, which demonstrate that the mechanism of apoptosis in the cell is closely related to the stress or shock response. This shock reaction triggers the process of induced cell death (Buckiová and Jelínek, 1995).

We repeatedly found a positive reaction in the centrilobule hepatocytes that rim the central vein of the liver lobe, which was much stronger when, compared with those in nearby hepatocytes. The cytoplasm especially reacted intensively with the anti-HSP70 antibody. In scientific articles concerned with intoxicant experiments, most intensive changes are described within the centrilobule space (Nanji et al., 1995). Endothelial cells, which line the central vein, also show positivity as do also the endothelial cells lining small arteries and veins of the portal space and the liver sinusoids. It can be supposed that a positive reaction is a consequence of the long-term irritation of these tissues/cells by toxins produced by the migrating parasites. Induction of increased synthesis of stress proteins caused by the activity of multiple toxins has already been described (Salminen et al., 1996, 1997).

Eosinophilia occurring during allergic or parasitic diseases is characterised by considerable eosinophil infiltration. Simon (1997) demonstrated that delayed eosinophil apoptosis is a mechanism causing tissue eosinophilia.

Eosinophils strongly infiltrating the connective tissue of liver suffering a parasitic invasion also show a positive reaction to anti-HSP70, in many cases. We presumed this positivity is related to specific granules but we considered this result to be inconclusive. On the other hand though, we cannot exclude that the greater occurrence of HSP70 may be related to their defensive function during parasitic diseases. The same conclusions are valid for the lymphocytes slightly infiltrating the connective tissue of the portal spaces.

To detect apoptotic cells on histology sections of the liver we used a simple staining method with toluidine blue and safranine. The method was created and described by Martin-Partido et al. (1986)) primarily for embryonic tissues.

Occasional hepatocytes, dispersed or accumulated in clumps, had many vacuoles in the cytoplasm and showed signs of cellular injuries. Such hepatocytes demonstrated remarkably positive immunoreaction. This reaction is probably not bound strictly to individual cells but to a certain cellular population. This can be explained by the different phases of the cell cycle and the intensity of proteosynthesis involved in the production of HSPs (Buckiová and Jelínek, 1995).

Shi et al. (1991) as also Arnold et al. (1996) were interested in the problem of the effects of the fixation reagent on the immunohistochemical reactions of the antigen-antibody and they both reached very similar conclusions. The review article of Arnold et al. (1996) especially described the effects of fixation on the following immunohistochemical detection of antigens during tissue processing. Their results indicated that in tissues fixed with formaldehyde (neutral buffered formaldehyde) using the routine immunohistochemical methods, it is possible to detect only a part of the antigen. Tissues fixed in formaldehyde and embedded in paraffin gave a poor immunohistochemical-staining signal. There is probably no optimal fixative for all antigens and the choice of fixative is usually a compromise depending upon both the antigen and the type of analysed tissue. This probably explains the weak reaction of stress proteins in the tissue when the concentration of the specific antibody has been changed. Shi et al. (1991) are also sure that success of the immunohistochemical detection of antibodies depends on the type and mode of fixation. Another explanation could also be the binding of HSP70 to the damaged proteins and these proteins

are probably degraded within the liver cell. Only exhaustion of the store of HSP70 in the cell permits a new synthesis of HSP70 and their binding to further proteins (Schiaffonati et al., 1990).

Another important factor that supports the release of HSP70 from newly synthesised proteins, is a hydrolysis of ATP. All studied HSP70 bind ATP with very high affinity. The affinity between HSP70 and the damaged proteins is increased with ADP and decreased with ATP (Palleros at al., 1991). A low relationship ATP. ADP increases the binding affinity of heat shock proteins-protein and blocks the release of HSP70 from the newly synthesised proteins.

It can be stated in conclusion that we tried in this study to demonstrate a narrow relationship between the acute pathological process of parasitical invasion and the synthesis of stress proteins in farm animals. Our conclusions correspond to similar research in clinical human pathophysiology.

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Czech Journal of Food Sciences (Potravinářské vědy)	6

DEMONSTRATION BY ELISA OF ANTIBODIES TO AUJESZKY DISEASE VIRUS IN PORCINE BLOOD SERUM AND MEAT JUICE

PRŮKAZ PROTILÁTEK PROTI VIRU AUJESZKYHO CHOROBY V KREVNÍM SÉRU A MASOVÉ TEKUTINĚ PRASAT ELISA METODOU

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ABSTRACT: Indirect ELISA allowing the demonstration of antibodies to Aujeszky disease virus (ADV) not only in blood serum but also in pork is described. A very high cytotoxicity of and approximately ten times lower concentrations of antibodies in meat juice must be taken into account in tests of pork. The cytotoxicity of meat juice for cell cultures is still apparent at dilutions 1 : 8 to 1 : 32 and consequently the use of virus neutralization test for the demonstration of antibodies in pork is limited to animals with very high titres of virus neutralizing antibodies (≥ 1 : 80). The high sensitivity and specificity of ELISA has allowed the demonstration of antibodies to ADV in meat of animals with marginal titres of virus neutralizing antibodies in blood serum (≤ 1 : 2) even after twelve-month storage at –20 °C. The State Veterinary Institutes in the Czech Republic have been using ELISA successfully for checks of swine herds and imported pork.

swine; Aujeszky disease virus; pseudorabies; ELISA; antibodies; blood serum; pork; meat juice

ABSTRAKT: Je popsána nepřímá metoda ELISA umožňující stanovit specifické ADV protilátky (Aujeszky disease virus antibodies) nejen v krevním séru, ale i ve výsekovém mase prasat. Při vyšetření vzorků masa se protilátky stanoví v masových šťávách, kdy je třeba počítat nejen s jejich vysokou cytotoxicitou, ale i s asi 10x nižší koncentrací protilátek ve srovnání s krevním sérem. Cytotoxicita masových šťáv pro buněčné linie se projevuje až do jejich ředění 1 : 8 až 1 : 32. Virusneutralizační (VN) test je proto ke stanovení antivirových protilátek v mase pouze omezeně použitelný. Z uvedených důvodů jím lze hodnotit jako pozitivní pouze vzorky masa odebrané od prasat s velmi vysokými titry sérových VN protilátek (VN ≥ 1 : 80). Dosažené výsledky potvrdily, že citlivost a specifita ELISA metody umožňuje spolehlivě stanovit antivirové protilátky i ve vzorcích masa odebraných od prasat s prahovými titry sérových VN protilátek (VN titry 1 : 2 a méně), a to i po jejich 12měsíčním uchovávání při teplotě –20 °C. ELISA metoda, dostupná ve formě komerční diagnostické soupravy, je již úspěšně využívána Státními veterinárními ústavy ke kontrole chovů prasat v České republice i importovaného masa.

prasata; virus Aujeszkyho choroby; pseudorabies; ELISA; protilátky; krevní sérum; vepřové maso; masová šťáva

INTRODUCTION

Aujeszky disease (pseudorabies) virus (ADV), a member of the family *Herpesviridae*, causes considerable losses particularly in large operations due to a high mortality in piglets and weaners. In older animals, the infection induces only milder clinical signs, such as fever, dysorexia, neurological disorders and pneumonia. However, the causative agent persists permanently in latent form in the host organism, in particular in tonsils and neural tissues (Sabó and Rajčáni, 1976; Šmíd et al., 1994). Debilitation or stress can lead to recrudescence of the infection associated with virus shedding. Latently infected animals present a permanent threat of infection and make the elimination of pseudorabies from swine herds a very difficult and expensive task that has not yet been completed in most European countries. Vaccines containing live avirulent strains of ADV failing to synthesise glycoprotein I (gI, gE, gp 63) are now available. Simultaneous tests by ELISA for anti-gI and anti-gII (anti-gIII) antibodies allow the distinction between infected and vaccinated animals and facilitate the elimination of pseudorabies from swine herds (Eloit et al., 1989; Motha and Ralston, 1991; Hefner et al., 1993; Stegeman et al., 1994).

Swine herds in the Czech Republic are currently free of pseudorabies and the use of any vaccines against this contagious disease is prohibited. It is not therefore necessary to distinguish between postinfection and postvaccination antibodies. Much more important under such conditions is to achieve maximum sensitivity and specificity of methods for the demonstration of antibodies to the complete set of ADV antigens.

Our aim was to develop a sensitive and specific method for the demonstration of antibodies to ADV not only in blood serum, but also in meat sample, that would allow

us to prevent the introduction of new ADV infections into local swine herds via imported chilled or frozen pork from countries from which the infection has not yet been eliminated.

MATERIAL AND METHODS

Animals

Six White Improved x Landrace weaners with live weights of 25 to 30 kg from an ADV free herd were used. After testing for the absence of antibodies to ADV, the animals received a single intramuscular injection of 5.0 ml inactivated vaccine against Aujeszky disease (Bioveta, Ivanovice na Hané, Czech Republic) and were slaughtered in groups of two 10, 14 and 21 days thereafter. Samples of diaphragmatic muscles, ham, loin, and picnic ham were collected immediately after slaughter and frozen at –20 °C. At the end of the experiment, the samples were tested for antibodies to ADV using the virus neutralization test (VNT) and ELISA. The same tests were done in the samples stored at –20 °C for 12 months.

Field samples

The following sets of blood serum and meat juice samples collected in the field were used:

a) twenty-five blood serum samples, collected earlier within the programme of elimination of Aujeszky disease from the Czech Republic and used for comparative trials of VNT and ELISA (10 samples collected from naturally infected pigs, 10 samples collected from pigs vaccinated with an inactivated vaccine, and 5 negative samples collected in an ADV-free herd);
b) eight samples of frozen pork (four samples of pork imported from France and Denmark and four coming from pigs slaughtered in the Czech Republic) supplied by cooling plants via the State Veterinary Institute, Brno, and District Veterinary Administration, Kroměříž, and tested by ELISA;
c) twenty-two samples of blood sera and meat juice, collected in Slovakia from slaughtered pigs immunised against Aujeszky disease with the live vaccine SUIVAK or the inactivated vaccine INAVAK (products of Mevak Nitra, Slovakia) and tested by ELISA and VNT at the State Veterinary Institute, Zvolen, Slovakia.

Processing of pork samples

Pork samples were prepared for examination using one of the following two procedures:

a) the samples were frozen and thawed. The released meat juice was stored at –20 °C;
b) the samples were cut into small pieces, mixed with buffered saline (1 ml per 1 g) and sea sand and homogenised in a mortar. The homogenate was centrifuged at 3 000 x g for 10 min and the supernatant was stored at –20 °C.

Virus neutralization test

Each sample of blood serum or meat juice was tested by the conventional virus neutralisation test (VNT) in twofold dilution series in quadruplicates using the cell line RK 13 and 100 $TCID_{50}$ of virus. The samples were filtered (Milipore, pore size 0.2 µ) and inactivated at 56 °C for 30 min before examination. The development of CPE was checked after 72 and 96 h of incubation at 37 °C in 5% carbon dioxide. Mean titres were calculated from the highest sample dilution still inhibiting the CPE.

Preparation of antigen for ELISA

The cell line RK-13 grown in Eagle's growth medium MEM supplemented with 5% bovine foetal serum was washed three times with the medium and infected with 0.1 multiplicity of infection of the ADV strain V-8 Plzeň. After 1 h adsorption at 37 °C and triple washing, the cell monolayer was further cultured in serum-free Eagle's medium. Control antigen (C-Ag) was prepared in the same way using a noninfected cell culture. After the CPE had fully developed in the infected cultures, the infected and control cells were frozen at –80 °C and thawed and cell debris was removed by centrifugation (3 000 x g for 15 min), the supernatant was transferred into bags for dialysis and its volume was reduced to 1/10 using polyethylene glycol 20 000 (Fluka). The concentrated suspensions of the viral (V-Ag) and the control (C-Ag) antigens were stored at –80 °C until use in ELISA.

Conjugate

Specific antibodies were isolated from caprine hyperimmune blood serum against porcine IgG by affinite chromatography (GASwIgG) and conjugated with horse radish peroxidase (POD) using the periodate method (Boorsma and Streefkerk, 1979). The conjugate (POD-GASwIgG) was diluted with PBS containing 0.05% Tween and 1% lactalbumin hydrolysate (PBST-LAH).

Chromogen

Working dilution of substrate was prepared by mixing 0.1 ml stock solution of chromogen (10 mg 3,3',5,5' tetramethyl benzidine in 1 ml dimethyl sulphoxide) with 11.0 ml 0.1 M acetate buffer, pH 5.8, and 10 µl 6% hydrogen peroxide immediately before use. Volumes of 100 µl were pipetted into each well.

The reaction was stopped after 10 to 30 min by adding equal volume of 1 M sulphuric acid.

Procedure and evaluation

The samples were tested in pairs of wells, one of which was coated with V-Ag and the other with C-Ag. Serial twofold dilutions were prepared with PBST-LAH starting from 1 : 40 for blood sera and 1 : 5 or 1 : 10 for meat juice. If present in the sample, antibodies to ADV reacted with V-Ag, and the reaction was detected during incubation (1 h at 37 °C) with POD-GASwIgG and visualised during the subsequent incubation with the substrate solution. Positive samples were identified by deeper colour in the wells containing V-Ag. Negative samples yielded identical and very weak colour reactions in both wells. Absorbancy was measured at 450 nm and the results were expressed in terms of net absorbancy (NA), i.e. difference in absorbancy between the wells containing V-Ag and C-Ag, respectively. The samples were scored as positive if NA > 0.2 and as negative if NA < 0.1. Samples with NA = 0.1 to 0.2 were scored as doubtful. The titration end point was the dilution yielding NA > 0.1.

RESULTS

Virus neutralization test

Six pigs from an ADV-free herd were tested by VNT and ELISA before and at various intervals after the vaccination. Antibody titres in the samples collected on post-vaccination days 10, 14, and 21 are presented in Tab. I showing that, in the pigs No. 1, 4, 5, and 6, CPE was completely inhibited in all the four wells only at the basic dilution 1 : 2 and in some wells at the dilution 1 : 4, i.e. at the VNT sensitivity limit.

The results for meat juice samples could not be evaluated owing to a high cytotoxicity of the samples at dilutions 1 : 8 to 1 : 32. Therefore VNT was aban-

I. ELISA antibodies to ADV in blood serum and meat juice of experimentally vaccinated pigs; samples stored for one year at –20 °C

Pig No.	Days after vaccination	VN titre ID_{50}	ELISA absorbance								
			blood serum 1 : 40				meat juice 1 : 10				
			V-Ag	C-Ag	NA*	score/titre**	sample***	V-Ag	C-Ag	NA*	score**
1	10	1 : 2.8	0.427	0.093	0.334	+	H	1.017	0.927	0.090	–
						1 : 40	P	1.021	0.816	0.205	+
							L	0.911	0.769	0.142	±
							D	0.981	0.712	0.269	+
2	10	1 : 5.6	0.658	0.087	0.571	+	H	1.273	1.075	0.198	±
						1 : 640	P	1.258	0.987	0.271	+
							L	1.306	1.110	0.196	±
							D	1.424	1.084	0.340	+
3	14	1 : 12.3	1.224	0.214	1.010	+	H	0.935	0.657	0.278	+
						1 : 1 280	P	1.365	1.190	0.175	±
							L	1.121	0.652	0.469	+
							D	1.373	1.135	0.238	+
4	14	1 : 3.14	0.771	0.105	0.666	+	H	0.958	0.747	0.211	+
						1 : 320	P	1.462	1.252	0.210	+
							L	1.017	0.785	0.232	+
							D	1.257	0.801	0.456	+
5	21	1 : 2.8	1.094	0.095	0.999	+	H	1.260	0.823	0.437	+
						1 : 320	P	1.023	0.451	0.572	+
							L	1.094	0.803	0.291	+
							D	1.075	0.426	0.649	+
6	21	1 : 3.14	1.270	0.099	1.171	+	H	1.095	0.892	0.203	+
						1 : 640	P	1.189	1.051	0.138	±
							L	1.026	0.981	0.045	–
							D	1.222	0.828	0.394	+

* = net absorbancy (difference between absorbancy in wells coated with V-Ag and wells coated with C-Ag)
** = samples scored as positive if NA > 0.2, doubtful if NA = 0.1–0.2, and negative if NA < 0.1
*** = sampling cuts: H – ham; P – picnic ham; L – loin; D – diaphragm

doned in the examination of further pork samples supplied by cooling plants or collected in distributive channels. Field samples of blood serum collected in Slovakia showed VN titres in the range 1 : 2 to 1 : 16 (Tab. II). Like in the previous set of samples, VN antibodies could not be demonstrated in meat juice owing to the cytotoxicity of the latter.

ELISA

a) Samples of blood sera and meat juice of the experimentally vaccinated animals (single administration of inactivated vaccine) were tested at the end of the experiment and after 12 months of storage at –20 °C with intermittent thawing. Antibodies to ADV were demonstrated in all the samples of both blood serum and meat juice. The data in Tab. I shows that the ELISA titres were apparently more specific and stable than VN antibody titres which decreased towards the end of the observation period (post-vaccination day 21).
b) Antibodies to ADV were demonstrated in all samples of blood serum and meat juice of pigs immunised against Aujeszky disease with the live vaccine SUIVAK (No. 1 through 7) or the inactivated vaccine INAVAK (No. I through XV) (Tab. II).
c) Comparative examinations of twenty-five blood serum field samples by ELISA and VNT showed a high correlation of results of the two tests ($r = 0.89$) as observed also in our previous investigations (Rodák et al., 1985).
d) No antibodies to ADV were demonstrable in any of the eight samples of frozen pork (imported or local) supplied by cooling plants.

DISCUSSION

The results have shown that ELISA is a reliable tool for the demonstration of antibodies to ADV not only in porcine blood serum, but also in meat juice of pork. The antibodies were also demonstrable in meat and meat juice kept at –20 °C for 12 months with intermittent thawing. ELISA is then suitable also for testing of frozen meat stored for long periods.

The applicability of VNT to the demonstration of antibodies to ADV in meat is limited owing to a high

II. ELISA antibodies to ADV in field samples of blood serum and meat juice of pigs vaccinated with live or inactivated vaccine

Pig No.	Vaccine	VN titre of blood serum	Absorbancy							
			blood serum				meat juice. +			
			V-Ag	C-Ag	NA*	score**	V-Ag	C-Ag	NA*	score**
1	live (SUIVAK)	1 : 8	1.903	0.707	1.196	+	1.644	0.495	1.149	+
2		1 : 8	2.035	0.869	1.166	+	2.055	1.796	0.259	+
3		1 : 8	2.041	0.995	1.046	+	2.053	1.115	0.938	+
4		1 : 2	1.713	1.184	0.529	+	1.950	1.213	0.737	+
5		1 : 4	2.113	0.360	1.753	+	2.056	0.835	1.221	+
6		1 : 4	2.027	0.427	1.600	+	1.871	0.629	1.242	+
7		1 : 2	1.745	0.845	0.900	+	1.729	0.390	1.339	+
I	inactivated (INAVAK)	1 : 16	2.110	0.336	1.774	+	2.076	0.281	1.795	+
II		1 : 4	2.075	0.293	1.782	+	1.959	0.110	1.849	+
III		1 : 8	2.102	1.420	0.682	+	1.967	0.472	1.495	+
IV		1 : 8	2.102	1.655	0.447	+	2.045	0.750	1.295	+
V		1 : 4	2.218	1.009	1.209	+	2.060	0.516	1.544	+
VI		1 : 2	2.242	1.069	1.173	+	2.109	0.536	1.573	+
VII		1 : 4	2.129	1.068	1.061	+	2.023	0.373	1.650	+
VIII		1 : 4	2.208	0.686	1.522	+	1.764	0.419	1.345	+
IX		1 : 8	2.175	0.597	1.578	+	2.009	0.436	1.573	+
X		1 : 2	2.161	0.781	1.380	+	1.383	0.135	1.248	+
XI		1 : 8	2.102	0.126	1.976	+	1.888	0.190	1.698	+
XII		1 : 4	2.102	1.056	1.046	+	1.902	0.661	1.241	+
XIII		1 : 4	2.218	0.525	1.693	+	1.955	0.699	1.256	+
XIV		1 : 4	2.160	0.574	1.586	+	1.547	0.297	1.250	+
XV		1 : 4	2.152	0.552	1.600	+	1.670	0.331	1.339	+

* = net absorbancy (difference between absorbancy in wells coated with V-Ag and wells coated with C-Ag)
** = samples scored as positive if NA > 0.2; meat juice samples could not be tested by VN test owing to a high cytotoxocity up to dilutions 1 : 8 to 1 : 32

cytotoxicity of meat juice up to dilutions 1 : 8–1 : 32, and to the approximately ten times lower concentrations of the antibodies in meat juice than in blood serum, as demonstrated also by Nielsen et al. (1998). It follows that even at minimum cytotoxicity of meat juice (evident only at dilution 1 : 8) containing ten times less antibodies to ADV than blood serum, VNT can identify positive meat samples only in animals with blood serum VN antibody titres 1 : 80.

Our experiments have demonstrated that ELISA detects the antibodies to ADV in meat juice samples collected from animals with VN titres as low as 1 : 2 or even below the limit of sensitivity of VNT.

Aujeszky disease control programmes of a number of EU countries include the vaccination with live avirulent gI^- strains of ADV and the use of ELISA with monoclonal antibodies, allowing the distinction between postvaccination and postinfection immune responses, for checks of efficacy of the programmes and of animals intended for transport. Such control programmes require a long lasting immunisation of all swine herds within a defined region, completed with other strict measures and elimination of serologically positive (gI^+) animals (Stegeman et al., 1994; Bol et al., 1998; Cavirani et al., 1998; Van Nes et al., 1998). Uncontrolled penetration of vaccine strains of herpesviruses into nonvaccinated herds has been described (Grom et al., 1994; Ros Bascunana et al., 1994; Schynts et al., 1998) and the danger of recombination among individual herpesvirus strains and reconversion into a virulent form must also be considered. Therefore, the policy of the countries that are free of pseudorabies must be very cautious. According to EU regulations, breeding animals older than 4 months intended for export into EU countries that are free of AD and in which vaccination against this infection is prohibited (such as Finland) must be free of ELISA antibodies to the complete set of ADV antigens (J. Offic. Commun. Europ., No. L 138/43).

Aujeszky disease was eradicated from the Czech Republic towards the end of 1988. The staff of the Veterinary Research Institute, Brno, contributed significantly to this success by extensive epizootiological investigations, development of diagnostic methods and assistance in their implementation, and active participation in the eradication programme (Rodák et al., 1985, 1986, 1987; Šmíd et al., 1981, 1985, 1994; Valíček et al., 1986, 1987). Our first-rate interest therefore is to maintain this favourable situation and to prevent any penetration of ADV into local swine herds. A highly sensitive and highly specific diagnostic method is indispensable to achieve this goal.

The results of our experiments have shown that the ELISA procedure described in this paper meets the requirements for sensitivity and specificity. Its merits have been confirmed by a comparison of ELISA and VNT results and by the demonstration of low concentrations of antibodies to ADV in meat of swine with marginal titres of VN antibodies in blood serum. The commercial diagnostic kit AD Ab ELISA, manufactured by Test-Line, Brno, Czech Republic, has been used successfully by State Veterinary Institutes of the Czech Republic for routine checks of the AD-free status of the swine population and checks of imported swine and pork.

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RATE OF NEUROLOGIC RECOVERY AS AN INDICATOR OF LONG-TERM PROGNOSIS IN DOGS WITH SURGICALLY TREATED THORACOLUMBAR DISC DISEASE

RYCHLOST OBNOVY NEUROLOGICKÝCH FUNKCÍ JAKO UKAZATEL DLOUHODOBÉ PROGNÓZY U PSŮ S CHIRURGICKY LÉČENÝM ONEMOCNĚNÍM TORAKOLUMBÁLNÍCH MEZIOBRATLOVÝCH PLOTÉNEK

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ABSTRACT: The time needed to regain the ability to walk without assistance was examined in 266 dogs, from the clinical group of 300 dogs with thoracolumbar disc disease treated by decompressive hemilaminectomy with removal of extruded disc material from the vertebral canal at the Clinic of Surgery and Orthopedics at University of Veterinary and Pharmaceutical Sciences in Brno during a four year period (April 1994 through March 1998). The time needed until complete recovery from TL-IVDD in 177 dogs in which the final outcome was graded as excellent was also evaluated. The outcome of surgical treatment was assessed based on 9 to 51 months follow-up. The time until dogs regained the ability to walk without assistance and until complete recovery (excellent outcomes only) differed (Mann-Whitney *U*-test; $p < 0.01$) between the groups of patients (grade II, III, IV A, IV B and IV C). Evaluating the time needed to regain the ability to walk without assistance in relation to the possible prediction of complete recovery (patients with excellent result of therapy compared to patients with very good and fair outcome of surgery) in dogs with grade IV A paraplegia a significant difference (Mann-Whitney *U*-test; $p < 0.01$) was found between patients with very good or fair outcomes and those with excellent outcomes. In grade IV A dogs, which underwent surgery within 48 hours after the onset of clinical signs, the following results were found: dogs with excellent outcomes regained the ability to walk in 1.70 ± 1.14 weeks after surgery, and dogs with very good or fair outcomes regained the ability to walk in 2.90 ± 1.47 weeks after surgery. In grade IV A dogs which underwent surgery more than 48 hours after the onset of signs, dogs with excellent outcomes regained the ability to walk in 2.15 ± 1.69 weeks after surgery, and those with very good or fair outcomes regained the ability to walk in 3.96 ± 2.29 weeks. Based on these results, the time in which a grade IV A dog regains the ability to walk unassisted is an objective prognostic indicator with a reliability of 95% ($\sigma_{\Delta 0.95}$) for those operated on within 48 hours from the onset, and also those operated on later than in 48 hours if appropriate decompressive surgery is performed.

intervertebral disc disease; dog; hemilaminectomy; prognosis

ABSTRAKT: Doba potřebná k opětnému obnovení schopnosti chůze bez pomoci majitele byla sledována u 266 psů, vybraných ze souboru 300 pacientů s onemocněním torakolumbálních meziobratlových plotének léčených dekompresní hemilaminektomií na Klinice chirurgie a ortopedie Veterinární a farmaceutické univerzity v Brně v průběhu čtyř let (duben 1994 až březen 1998). U 177 psů, u nichž bylo dosaženo výborného výsledku léčby, jsme zjišťovali dobu nezbytnou k úplné obnově všech neurologických funkcí. Výsledek chirurgické léčby vycházel ze sledování klinického stavu pacientů po dobu 9 až 51 měsíců od operace. Časový interval mezi dekompresní operací a okamžikem opětného obnovení schopnosti samostatné chůze, respektive kompletní obnovy všech neurologických funkcí, se lišil (Mann-Whitney *U*-test; $p < 0{,}01$) u jednotlivých skupin pacientů (stupeň II, III, IV A, IV B a IV C). Hodnocení vztahu mezi dobou potřebnou k obnově chůze bez pomoci majitele a možnou prognózou úplné obnovy neurologických funkcí u paraplegických pacientů stupně IV A odhalilo signifikantní rozdíly ($p < 0{,}01$) mezi pacienty s velmi dobrým či uspokojivým výsledkem léčby a pacienty s výborným pooperačním výsledkem. Psi s paraplegií IV A operovaní do 48 hodin po jejím vzniku, u kterých byl výsledek léčby označen jako výborný, začali znovu bez pomoci chodit v průměru za $1{,}70 \pm 1{,}14$ týdne, zatímco psi s velmi dobrým či uspokojivým výsledkem léčby byli schopni chůze za $2{,}90 \pm 1{,}47$ týdnů po chirurgickém zákroku. Pacienti stupně IV A operovaní za více než 48 hodin po vzniku paraplegie, u kterých došlo k úplné obnově neurologických funkcí (výborný výsledek), začali chodit v průměru za $2{,}15 \pm 1{,}69$ týdnů po dekompresi. Jedinci s velmi dobrým či uspokojivým výsledkem léčby byli schopni chodit až po $3{,}96 \pm 2{,}29$ týdnech od operace. Na základě těchto výsledků lze říci, že doba potřebná k opětnému obnovení schopnosti chůze po správně provedené dekompresní hemilaminektomii je u pacientů s paraplegií stupně IV A (operovaných jak do 48 hodin, tak po více než 48 hodinách od vzniku paraplegie) objektivním prognostickým ukazatelem konečného výsledku léčby.

onemocnění meziobratlových plotének; pes; hemilaminektomie; prognóza

INTRODUCTION

Thoracolumbar (TL) intervertebral disc herniations are the most common cause of paralysis in dogs. Disc lesions in the thoracolumbar region of the spine represent from 84 to 86% of the clinical cases of intervertebral disc disease (IVDD) seen in dogs (Gage, 1975; Hoerlein, 1978). The age incidence for clinical manifestation of the disease in chondrodystrophoid breeds is highest at 3 to 6 years (Toombs and Bauer, 1993). Males and females are approximately at equal risk (Hoerlein, 1953). The severity and duration of neurological deficits, as well as the method of therapy, are correlated with outcome (Nečas, 1995, 1999). There are controversies about the treatment of TL-IVDD with regard to the necessity for decompressive surgery, the type of decompressive surgery, the necessity to remove the compressive mass of extruded disc material, and the therapeutic value of disc fenestration (Henry, 1975; Denny, 1978; Bitetto and Thacher, 1987; Walker and Betts, 1985; Black, 1988; Jeffery, 1988; Butterworth and Denny, 1991; Davies and Sharp, 1983; McKee, 1992; Yovich et al., 1994). Hemilaminectomy is the most commonly used surgical procedure for spinal cord decompression and removal of herniated disc material from the vertebral canal (Cook, 1992).

The prognosis for neurological recovery after treatment is dictated by the severity of injury to the spinal cord. To provide a prognosis before surgical exploration and visualization of the spinal cord, the clinical history and neurological examination are used. We believe that there are additional and more objective prognostic indicators that should also be used before surgery as changes in the creatine kinase and lactate dehydrogenase activities in cerebrospinal fluid of dogs with thoracolumbar disc disease (Nečas and Sedláková, 1999).

The purpose of this study was to find a new and different objective prognostic indicator for neurological outcome in dogs with grade IV A paraplegia due to TL-IVDD. The time needed until these dogs regained the ability to walk without assistance was evaluated as a possible long-term prognostic indicator for those operated on within 48 hours from the onset, and also those operated on later than in 48 hours. The longest time needed to regain the ability to walk without assistance after the surgical procedure (according to which it was possible to predict a complete recovery) was assessed as $\sigma_{\Delta 0.95}$ (mean + 2 sd).

MATERIAL AND METHODS

The time needed to regain the ability to walk without assistance was examined in 266 dogs, from the clinical group of 300 dogs with TL-IVDD at the Clinic of Surgery and Orthopedics at University of Veterinary and Pharmaceutical Sciences in Brno during a four year period (April 1994 through March 1998). Dogs were treated by hemilaminectomy with removal of extruded disc material from the vertebral canal. Prophylactic fenestrations of other discs at the risk region of the thoracolumbar spine (from T11–T12 to L3–L4) were not performed. Nineteen of these 300 patients died or were euthanatized during the perioperative or early postoperative period, and fifteen dogs never regained the ability to walk after surgery, and were lost for a follow-up.

Each dog received standard diagnostic evaluation, including neurological examination. These 266 dogs were classified into groups according to severity of signs (Tab. I) (Toombs and Bauer, 1993). Ten of them elicited neurological signs of grade II involvement, 68 dogs grade III, 159 dogs grade IV A, 24 dogs grade IV B, and 5 dogs grade IV C. The duration of clinical signs was defined as the interval between the appearance of initial neurological deficit, which is typical of each group (i.e., grade III, grade IV A, grade IV B etc.) of the patients, and surgery. In cases of slow progression of paraplegia (loss of all pelvic limb sensory and motor function), paraparesis (still purposeful movements in the hind limbs), which preceded a plegia, was not considered as a clinical sign typical of the grade. The general anesthesia protocol used in a given patient was dependent upon its general condition (Paddleford and Erhardt, 1992; Wooten and Lowrie, 1993; Thurmon et al., 1996). Survey radiographic examination and myelography, using transmedullary lumbar puncture (Barber et al., 1987)

I. Classification of dogs with TL-IVDD according to severity of clinical signs

Grade	Clinical manifestation	Treatment options
I	first episode of back pain and no neurological deficit	M or F
II	recurrent pain and/or mild to moderate paraparesis	M, F, D, D + F
III	severe paraparesis	D or D + F
IV	paraplegia	
A	with deep pain intact	D or D + F
B	deep pain absent 48 hours D + d	
C	deep pain absent 48 hours	M or D + d

Explanatory notes:
M = medical therapy, F = disc fenestration, D = decompressive surgery and removal of extruded disc material, d = durotomy (modified and reprinted with permission from Toombs and Bauer, 1993)

II. The time needed to regained ability to walk without assistance (mean ± sd) in relation to the severity of clinical signs in 266 dogs with TL-IVDD

Severity of clinical signs*	Number of patients A = 266	The time needed to regained ability to walk (weeks) mean ± sd
Grade II	10	1.70 ± 2.21
Grade III	68	1.59 ± 1.01
Grade IV A	159	2.34 ± 1.71
Grade IV B	24	5.46 ± 4.02
Grade IV C	5	5.80 ± 8.04

* Classification of dogs into groups according to severity of clinical signs.

III. The time (mean ± sd) required for complete recovery (excellent outcome) in relation to the severity of clinical signs in 177 dogs with TL-IVDD

Severity of clinical signs*	Number of patients A = 177	The time needed to complete recovery (weeks) mean ± sd
Grade II	8	5.00 ± 5.71
Grade III	55	5.82 ± 7.77
Grade IV A	105	7.63 ± 12.83
Grade IV B	7	9.57 ± 6.32
Grade IV C	2	10.00 ± 0.00

* Classification of dogs into groups according to severity of clinical signs.

between L5 and L6 vertebrae, were performed prior to surgery.

Searching for necessary data we used clinical records on hospitalised patients, and outpatients, respectively. A long-term follow up was obtained by examination of the dog at our hospital when feasible, and by a telephone conversation with the owner when not. Owners were asked to estimate the time needed until their dogs regained the ability to walk without assistance and to describe the most complete extent of recovery that was evident in the dog and the time in which this was noted following surgery. Residual weakness and/or incoordination, and recurrence of back pain, paresis or paralysis were also recorded. Patients were observed at least 9 months after surgery (9–51 months follow-up) and results of the therapy were classified as excellent (complete recovery of motor and urinary function), very good (minimal motor deficit when walking on a slippery surface, and complete recovery of urinary bladder function), fair (obvious remaining dysfunction, either motor or urinary, but animal retained independent function and usefulness) and poor (not enough improvement to be returned to the owner as an independent animal).

The time needed until complete recovery from TL-IVDD in 177 dogs in which the final outcome was graded as excellent was also evaluated.

The longest time to regain the ability to walk without assistance (according to which it is possible to predict complete recovery) was assessed as $\sigma_{\Delta 0.95}$ (mean + 2 sd).

Means and standard deviations were calculated for all variables. The values were analyzed using Mann-Whitney *U*-test. Statistical analyses were done using Stat plus 1.10 (Matoušková et al., 1992).

RESULTS

Twenty-four different breeds of dogs, including mongrels, were represented in 300 clinical cases of thoracolumbar disc disease. The most commonly affected breeds in our study were: Dachshund (71.00 %), Mongrel (8.67%), Pekingese (4.67%), Cocker Spaniel (2.67%), Miniature Schnauzer (1,67%), Poodle (1.67%), French Bulldog (1.67%), Lhasa-apso (1.33%), Basset hound (1.00%), American Cocker (0.67%) and Shih-tzu (0.67%). The age of the dogs varied from 2 to 13 years with an average of 6.82 1.58 years. One hundred seventy-two dogs (57.33%) were males and one hundred twenty-eight (42.67%) were females. The rate of neurological recovery was different in each group of dogs according to the severity of clinical signs (grades II, III, IV A, IV B and IV C) (Nečas, 1999).

Two hundred sixty-six cases were identified as appropriate subjects for this portion of the study. All of these dogs regained the ability to walk after surgical intervention and had complete and long-term follow-up information available in the medical record. The variables concerning the complete recovery from TL-IVDD were evaluated in 177 dogs with excellent outcomes.

The time needed until dogs regained the ability to walk without assistance (Tab. II) and until complete recovery (in dogs with excellent outcomes) (Tab. III) differed between the groups of patients. The mean time after which dogs with grade II involvement were able to walk without the owner's assistance was 1.70 ± 2.21 weeks. The time of complete recovery from neurological deficits in these dogs varied from 1 to 16 weeks, with the average of 5.00 ± 5.71 weeks. The mean time after which dogs with grade III involvement were able to walk without the owner's assistance was 1.59 ± 1.01 weeks (1 to 4 weeks) and the mean time until complete recovery in these dogs was 5.82 ± 7.77 weeks (1 to 50 weeks). In dogs with grade IV A involvement, the ability to walk was seen from 1 to 10 weeks following surgery, with an average of 2.34 ± 1.71 weeks. Complete recoveries in these dogs were achieved within 7.63 12.83 weeks after hemilaminectomy (1 to 100 weeks). When selecting only those patients with duration of grade IV A paraplegia less than 48 hours, the ability to walk was regained within 2.03 ± 1.34 weeks after surgery (1 to 8 weeks) and complete recovery was evident in 6.81 ± 14.12 weeks following the procedure (1 to 100 weeks).

The same variables in dogs with a duration of IV A paraplegia which exceeded 48 hours were as follows: 3.02 ± 2.18 weeks (1 to 10 weeks), and 8.32 11.73 weeks (1 to 70 weeks), respectively. The mean time in which dogs with grade IV B involvement regained the ability to walk without assistance was 5.46 ± 4.02 weeks following the surgery (1 to 16 weeks). The time until complete recovery varied from 3 to 20 weeks, with an average of 9.57 ± 6.32 weeks after surgery. Dogs with grade IV C involvement regained the ability to walk in 5.80 ± 8.04 weeks after surgery (1 to 20 weeks) and those with the excellent outcomes recovered completely in 10.00 ± 0.00 weeks after surgery.

Assessing the time needed to regain the ability to walk without assistance in relation to severity of clinical signs before surgery, a significant difference was seen between grade III dogs (paraparesis) and grade IV A dogs (paraplegia; deep pain intact) using Mann-Whitney *U*-test ($p < 0.01$). The results of the statistical analysis showed significant differences ($p < 0.01$) in the time necessary to regain the ability to walk between dogs of grade IV A (deep pain intact; operated on within 48 hours after the onset of clinical signs) and dogs of grade IV B (no deep pain sensation up to 48 hours). Significant differences ($p < 0.01$) were found between patients of grade IV A (deep pain intact; operated on more than 48 hours after the onset of clinical signs) and those of grade IV C (no deep pain perception more than 48 hours). Comparing the time needed to walking without assistance significant differences ($p < 0.01$) between dogs of grade IV A operated on within 48 hours after the onset of paraplegia and those operated on later than in 48 hours were found. No differences were found when paretic dogs of group II were compared with those of group III.

The time needed to achieve complete recovery in dogs with excellent outcomes was significantly different (Mann-Whitney *U*-test; $p < 0.01$) between grade II dogs (mild to moderate paraparesis) and grade III dogs (severe paraparesis). Similar differences ($p < 0.01$) were also evident between grade III dogs (paraparesis) and grade IV A dogs (paraplegia with deep pain intact). Significant differences ($p < 0.01$) were also seen in the time required for complete recovery between grade IV A dogs (paraplegia with deep pain intact; operated on within 48 hours after the onset of clinical signs) compared to grade IV B dogs (paraplegia with no deep pain sensation up to 48 hours). Similar differences ($p < 0.01$) were found between paraplegic dogs of grade IV A (deep pain intact; operated on more than 48 hours after the onset of clinical signs) and those of grade IV C (no deep pain perception more than 48 hours). Significant differences ($p < 0.01$) in the time needed to achieve complete recovery were also seen between dogs of grade IV A operated on within 48 hours after the onset of paraplegia and those operated on later than in 48 hours.

Evaluating the time needed to regain the ability to walk without assistance in relation to the possible prediction of complete recovery (patients with excellent result of therapy compared to patients with very good and fair outcome of surgery) in dogs with grade IV A paraplegia (Tab. IV) a significant difference (Mann-Whitney *U*-test; $p < 0.01$) was evident between patients with very good or fair outcomes and those with excellent outcomes. In grade IV A dogs, when surgery was performed up to 48 hours after the onset of signs, a significant difference ($p < 0.01$) was found between dogs with very good or fair outcomes and those with excellent outcomes. When surgery was performed more than 48 hours after the onset of signs in group IV A dogs, a similar difference was found between dogs with very good or fair outcomes and those with excellent outcomes ($p < 0.01$). Dogs with grade IV A paraplegia which recovered completely (excellent outcomes), regained the ability to walk in 1.81 ± 1.30 weeks after surgery. Dogs with grade IV A paraplegia with very good or fair outcomes, regained the ability to walk in 3.37 ± 1.94 weeks after surgery. Evaluation of the same parameters in grade IV A dogs which underwent surgery within 48 hours after the onset of clinical signs, revealed the following results: dogs with excellent outcomes regained the ability to walk in 1.70 ± 1.14 weeks after surgery, and dogs with very good or fair outcomes regained the ability to walk in 2.90 ± 1.47 weeks after surgery. In grade IV A dogs which underwent surgery more than 48 hours after the onset of signs, dogs with excellent outcomes regained the ability to walk in 2.15 ± 1.69 weeks after surgery, and those with very good or fair outcomes regained the ability to walk in 3.96 ± 2.29 weeks.

IV. The time (mean ± sd) needed to regain the ability to walk in dogs with IV A paraplegia with excellent, and very good/fair outcomes according to the duration of clinical signs

Dogs of grade IV A* $n = 159$	Number of patients $n = 105$	Time needed to regain the ability to walk (weeks) mean ± sd excellent outcome	Number of patients $n = 54$	Time needed to regain the ability to walk (weeks) mean ± sd very good + fair outcome
IV A paraplegia up to 48 hours	79	1.70 ± 1.14	30	2.90 ± 1.47
IV A paraplegia more than 48 hours	26	2.15 ± 1.69	24	3.96 ± 2.29
Total IV A	105	1.81 ± 1.30	54	3.37 ± 1.94

* Classification according to duration of clinical signs.

DISCUSSION

Dogs in this study represent a typical sample of clinical patients, among which dachshunds predominate (71%). The age of the dogs (mean 6.82 ± 1.58 years) was similar to that in previous reports (Gage, 1975; Hoerlein, 1978; Toombs and Bauer, 1993).

Based upon our results, it is possible to conclude that useful correlations exist between the time needed to regain the ability to walk unassisted, the severity of neurological deficits, and duration of these signs at the time of presentation. Similar correlations exist between the time needed to the complete recovery (excellent outcomes), the severity of neurological deficits, and duration of signs after onset.

The mean time within which these patients were able to walk without assistance was approximately 1 to 2 weeks after surgery in dogs with grades II or III involvement, 2 to 3 weeks in dogs wiht grade IV A involvement, and 5 to 6 weeks in dogs with grades IV B or IV C involvement (Tab. II).

Similarly it is possible to estimate the time until complete recovery (in cases which achieve excellent outcomes) which in our patients was 5 to 6 weeks in grade II or III dogs, 7 to 9 weeks in grade IV A dogs, and 9 to 10 weeks in grade IV B or IV C dogs (Tab. III). In predicting the eventual outcome for our surgical TL-IVDD patients the following guideline applies: the more severe the spinal cord injury, the longer the recovery from paraparesis/paraplegia.

The mean time within which grade IV A dogs regained the ability to walk was 1.70 ± 1.14 weeks in the subset of patients which had surgery within 48 hours and eventually achieved complete recovery. If similar patients underwent surgery later than in 48 hours after the onset of signs, this value increased to 2.15 ± 1.69 weeks. In our study, the longest time needed to regain the ability to walk without assistance after the surgical procedure, was accurately predicted by the likelihood of complete recovery ($\sigma_{\Delta 0.95}$). In grade IV A dogs this time was $\sigma_{\Delta 0.95} \leq 3.98$ weeks (mean + 2 sd) when decompressive surgery was performed within 48 hours from the onset of paraplegia, and $\sigma_{\Delta 0.95} \leq 5.53$ weeks when surgery was performed later than in 48 hours.

When describing the case prognosis to the owner, it is possible to predict, for example, that the dog with grade IV A paraplegia operated on within 48 hours after the onset of signs, which regains the ability to walk unassisted within 1.70 ± 1.14 weeks has a high likelihood of achieving complete recovery. This supposes no neurological deficits before this episode of transverse myelopathy and appropriate postoperative management (Hart et al., 1997; Jerram et al., 1997).

Conversely, grade IV A dogs, operated on within 48 hours from the onset which were able to walk unassisted within 2.90 ± 1.47 weeks after surgery ($\sigma_{\Delta 0.95}$ = 5.84 weeks), never recovered completely from paraplegia. Similarly, grade IV A dogs operated on later than 48 hours after onset, which were able to walk unassisted within 3.96 ± 2.29 weeks after surgery ($\sigma_{\Delta 0.95}$ = 8.54 weeks), never recovered completely from paraplegia.

Based on these results, the time in which a grade IV A dog regains the ability to walk unassisted is an objective prognostic indicator with a reliability of 95% ($\sigma_{\Delta 0.95}$) for those operated on within 48 hours from the onset, and also those operated on later than in 48 hours if appropriate decompressive surgery is performed.

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LUNG FILAROIDOSIS IN THE BEAGLE DOG BREEDING COLONY

PLICNÍ FILAROIDOSIS V CHOVNÉ KOLONII PSŮ PLEMENE BEAGLE

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ABSTRACT: Thirty-eight (2%) of a total of 1885 dogs autopsied at the Pathology Department of the Research Institute for Pharmacy and Biochemistry at Rosice nad Labem during a 15-year (1975–1989) period showed pathological alterations of a lungworm infection. According to findings of lung gross pathology and histopathology and of the parasite morphology, we supposed the *Filaroides* infection without any clinical significance. This is the first report of *Filaroides* infection in the Czech Republic.

lungworm *Filaroides*; histopathology; Beagle dog

ABSTRAKT: Mezi 1885 psy pitvanými na oddělení patologické anatomie Výzkumného ústavu pro farmacii a biochemii v Rosicích nad Labem se v letech 1975 až 1989 vyskytlo 38 jedinců (2 %) s nálezy plicní červivosti. Podle charakteru změn v plicní tkáni a podle morfologie zachycených parazitů usuzujeme, že se jedná o invazi metastrongylem rodu *Filaroides* bez klinického významu. Jde o první záchyt na území České republiky.

plicní nematod *Filaroides*; histopatologické nálezy; pes beagle

INTRODUCTION

Among many metazoan parasites, which either pass the canine lung or look for it as a target organ, three representatives of the genus *Filaroides* (*Nematoda: Filaroididae*) are found. In lungs they complete their life cycle as adults.

Filaroides osleri (Cobbold, 1879) was first described by Osler in the canine trachea and lung in 1877. Since that time, many cases (Mills and Nielsen, 1966; Mills, 1967; Kotani et al., 1995) have been recorded in dogs, coyotes, or wolves worldwide.

Filaroides milksi Whitlock, 1956 was recognized as the new species in dogs only in 1956 (Whitlock, 1956). Until now, several cases have been described in the U.S.A. (Whitlock, 1956; Jubb, 1960; Mills and Nielsen, 1966; Mills, 1967; Corwin et al., 1974; Sawyer et al., 1976) and in Japan (Yamamori et al., 1975, Yasuda et al., 1976), one case in Belgium (Cremers et al., 1978), and five cases in Great Britain (Plumb, 1981).

The female of this species is 9.2 to 11.4 mm long, 116 to 174 μm wide. Uteri of adult females are filled with embryonated ova. The male is 2.2 to 4.0 mm long, 50 to 101 μm wide. The body diameter of the first-stage larvae is 17.5 μm on average, their body length is 200 to 250 μm. A reddish-brownish finely granular pigment has been described positioned around the digestive tube in both larvae and adults (Mills, 1967; Corwin et al., 1974; Plumb, 1981). Its origin is related to the degradation of haemoglobin from ingested red blood cells (Whitlock, 1956). Larvae of this species elicit the conspicuous tissue reaction (Jubb, 1960).

The most recent, described in dogs in 1973, is the species *Filaroides hirthi* Georgi et Anderson, 1975. It has been revealed in several laboratory breeding colonies (Hirth and Hottendorf, 1973; Georgi and Anderson, 1975; Georgi et al., 1976; Waner et al., 1991; Bahnemann and Bauer, 1994; Crippa, 1995) or in massive to fatal infections in individual animals (Craig et al., 1978; August et al., 1980; Genta and Schad, 1984; Rubash, 1986; Valentine and Georgi, 1987; Pinckney et al., 1988) in the U.S.A., in several dogs in Australia (Beveridge et al., 1983) and Japan (Kagei et al., 1976a, b), and in isolated cases in Germany (Geisel, 1979), Turkey (Doganay, 1983), Great Britain (Spencer et al., 1985), Spain (Carrasco et al., 1997), and Ireland (Torgerson et al., 1997).

The female of *F. hirthi* is 6.6 to 13.0 mm long, 58 to 102 μm wide. Uteri of adult females are also filled with embryonated ova. The male is 2.3 to 3.2 mm long, 35 to 43 μm wide. The body length of the first-stage larvae is 150 to 190 μm. Fine granules of a refractive material are described in the larval digestive tube (Georgi and Anderson, 1975), sometimes unambiguously declared as being hemosiderine (Pinckney et al., 1988).

The life cycle of these three metastrongylid nematodes is direct without any intermediate host (Mills and Nielsen, 1966; Mills, 1967; Georgi, 1976, 1979, 1987;

Georgi et al., 1976, 1977, 1979a, b; Bahnemann and Bauer, 1994).

MATERIAL AND METHODS

During a period of 15 years, 1975–1989, 1 885 adult beagle dogs, 977 females and 908 males, were autopsied and histologically examined at the Pathology Department of the Research Institute for Pharmacy and Biochemistry. The dogs served as experimental animals for various drug safety-evaluating studies (1 725 animals on average 1.5 year old) or came as old individuals from the institute-breeding colony (160 animals 5 to 7 years old). Sires of this breeding colony were imported from Great Britain in the late sixties and early seventies.

Dogs were euthanatized with an *i.v.* overdose of Thiopental Spofa and completely autopsied. After gross pathology-evaluation, organ and tissue specimens were fixed in 10% neutral buffered formaldehyde. Histological slides were made by the common paraffin technique, sectioned at 6 µm, and stained with haematoxylin-erythrosin (HE). Indicated sections of the lung parenchyma were stained by methods after Perls for iron and after von Kossa for calcium deposits.

RESULTS

In 38 (2%; 19 males, 19 females) of the 1 885 dogs examined we detected adult worms in the lung parenchyma. None of the dogs involved suffered from clinically manifested breathing disorders. All 19 males and 17 females came from various drug safety-evaluating studies, 2 old females left the breeding colony. The age of animals varied within the range 9 months to 7 years with the average of 16 months.

Nine dogs (23.7% of dogs involved, 6 males and 3 females) had no gross pathology changes in lungs. Subpleural, red-violet foci with the diameter of 3 to 15 mm were found in 13 males and 13 females (68.4%) predominantly in medial and caudal lung lobes. Foci were mostly solitary but sometimes multiple. In three females (7.9%), solitary greyish small foci (1 to 2 mm) were found in the same localization. Histopathological findings could be classified in the three following groups.

The first group consisted of changes revealed in 4 males and 5 females (23.7%). Emphysematous areas with coiled masses of 1 to 7 intact adult worms, i.e. worm nodules or nests were found in the lung parenchyma. Female worms with embryonated ova in their uteri prevailed. Nests were mostly solitary with the exception of findings in 1 female dog, in which we found an intensive infection of 8 nests within 1 cm^2 of the tissue section. No inflammatory reaction occurred in the vicinity of these worm nodules (Figs. 1, 2).

The second group of 12 males and 10 females (57.9%) was featured with the presence of pneumonic or bronchopneumonic foci surrounding partially degraded parasite bodies. Embryonated eggs were also present in female worms' uteri. In 9 male dogs and 8 female dogs we found a varying number of such foci only. In 3 male dogs and 2 female dogs, the damage to lung parenchyma was combined with worm nodules as described in the first group. Inflammatory foci were of the various age, subchronic ones prevailed with the conspicuous presence of eosinophilic granulocytes. Mostly subpleural location of these foci was found. Some foci were encountered even in the depth of lung parenchyma following the bronchial ramification. In several cases, perivascular infiltrates were revealed consisting of lymphocytes and eosinophilic granulocytes. The central necrosis surrounding debris of parasite bodies occurred in 5 cases. The degradation of parasites manifested as the disintegration of their internal structures, loss of staining patterns, or calcifying. Especially in the latter case, the development of an epitheloid lining was encountered. The obliterating bronchiolitis was sometimes revealed in the vicinity of larger foci. Findings in this group were depicted in Figs. 3, 4, and 5.

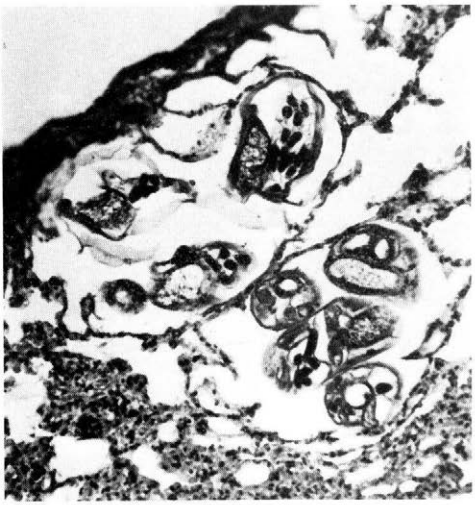
In the third group of 3 male dogs and 4 female dogs (18.4%), mostly calcified debris and remnants of the parasite bodies were limited by tuberculoid granulomas of varying number. The periphery of the epitheloid lining containing foreign body-giant multinucleated cells was surrounded by the newly formed lymphoid tissue in three cases. In one case, we revealed even a plastic reaction (Figs. 5, 6, 7).

The sections of both parasite bodies and their larvae were almost uniform in all our cases. The width of adult bodies varied in the range 44 to 110 µm (in most cases about 63 µm), the width of larval bodies was 8.5 to 10 µm. Beneath the cuticle of parasites, no conspicuous muscular layer was observed (Fig. 2); we found many first-stage larvae coiled in egg shells in the uteri of female worms. Released larvae did not show any marked excretory columns, their tails were S-shaped (Fig. 8). Some larvae did not provoke any inflammatory reactions; occasionally, the minimal tissue response was encountered in the vicinity of some larval bodies.

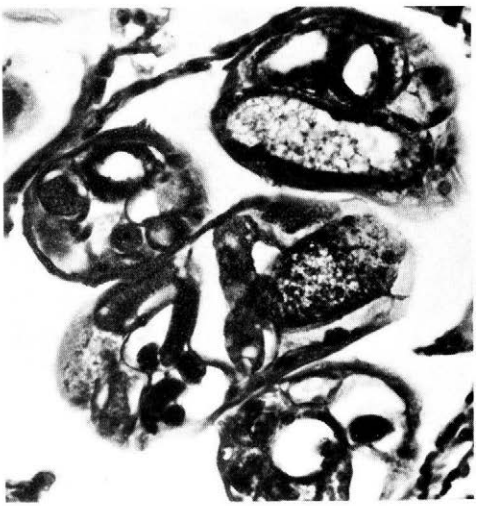
In seven cases with worm nodules without the inflammatory reaction and in eight cases with the inflammatory foci, we detected the presence of fine granulated-red-brown pigment in the wall of the worm digestive tube and/or in its vicinity or lumen. Sometimes the positive reaction of Perls' staining occurred. Such a pigment was never found in worm bodies in tuberculoid granulomas, perhaps because of advanced degradation.

DISCUSSION

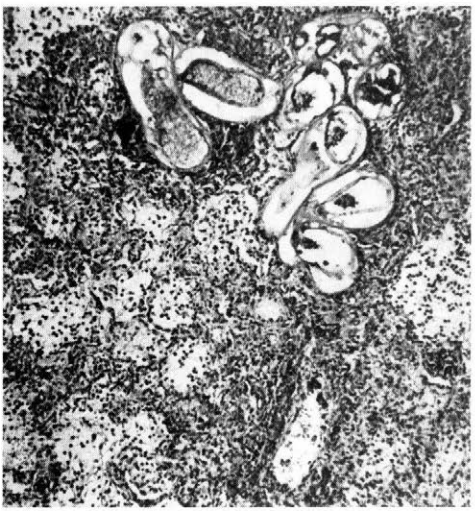
Both the body size and structure of lungworms and the reactions of the lung tissue that we found were in accordance with findings of both Hirth and Hottendorf (1973) and Mills and Nielsen (1966). The reaction of



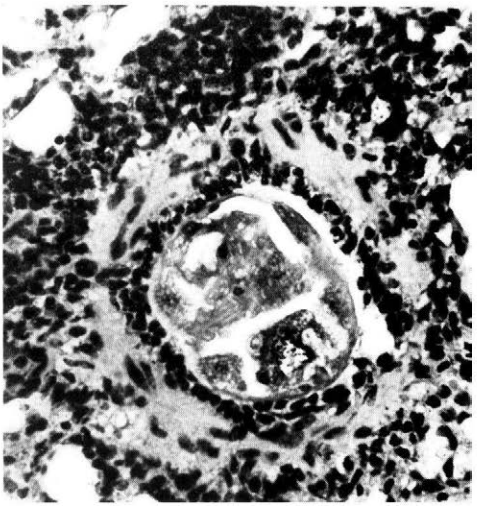
1. Subpleural, emphysematous node with sections through several adult female nematodes; haematoxylin-erythrosin, 25x



2. Gravid uteri of adult females containing embryonated ova; haematoxylin-erythrosin, 100x



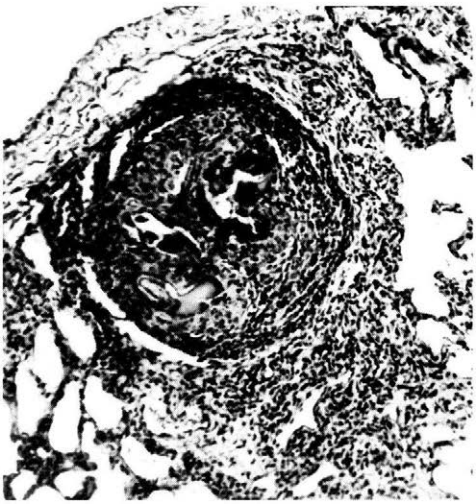
3. Exudative inflammation surrounding adult nematodes at the early stage of degeneration; haematoxylin-erythrosin, 20x



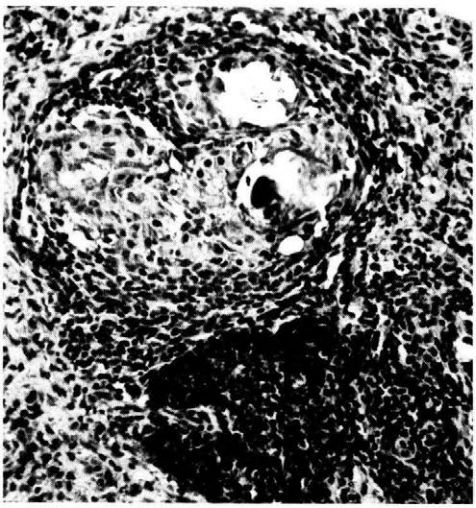
4. Degenerated nematode in the lumen of a bronchiole; haematoxylin-erythrosin, 72.5x

the lung tissue was raised by damaged or dead parasites or by some migrating larvae. The intact adult worms did not raise any reaction except forming the nest worm nodule. Features described were also in accordance with findings of Whitlock (1956), Mills and Nielsen (1966), Mills (1967), Georgi et al. (1976), Waner et al. (1991), and Crippa (1995). Therefore, a major part of described cases including our collection was found accidentally. According to Hirth and Hottendorf (1973), findings of nests without any inflammatory reactions were encountered in 25% of dogs involved. This was in a good accordance with our findings (23.7% of dogs involved).

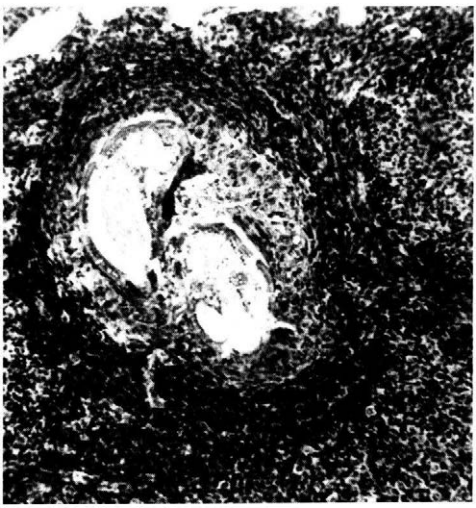
Georgi (1979) dealt in detail with distinguishing the species *Filaroides milksi* and *F. hirthi*. In our collection, the only hallmark – body diameter, was suitable. The tissue reaction to parasite larvae was mostly absent with a minimal tissue response in some cases. This was



5. Subpleural, granulomatous, foreign-body reaction to degenerated and calcified parasites; haematoxylin-erythrosin, 32x



6. Epitheloid lining of a calcified nematode within a granuloma; haematoxylin-erythrosin, 72.5x



7. Well-developed granulomatous, foreign-body reaction with the presence of germinal centres within surrounding lymphoid tissue; haematoxylin-erythrosin, 32x

8. Released larva in the lumen of a respiratory bronchiole; haematoxylin-erythrosin, 160x

in accordance with findings of Hirth and Hottendorf (1973) and Torgerson et al. (1997) and in contrast to findings of Jubb (1960). Thus, we arrived at the conclusion we found *F. hirthi* infection. However, methods used did not allow us to differentiate between *F. hirthi* and *F. milksi* safely.

Other canine lungworm infestations could be omitted because of quite different localization and features of changes in the lung tissue and/or sizes and features of parasites and their larvae. *F. osleri* infestation resulted in tracheal and bronchial nodules (Mills and Nielsen, 1966; Mills, 1967; Kotani et al., 1995). The lung dirofilariasis in dogs (Dyk and Zavadil, 1976; Atwell et al., 1988) involved pulmonary arteries, changes in the lung parenchyma were related to this primary damage. In rare *Crenosoma vulpis* infections, pathologic changes were found in bronchi and bronchi-

oles (Dyk and Zavadil, 1976; Georgi, 1987). *Capillaria aerophila* was encountered in nasal cavity, trachea, and bronchi (Dyk and Zavadil, 1976; Georgi, 1987). *Angiostrongylus vasorum* larvae had a dorsal tail spine and were longer (Torgerson et al., 1997).

The clinical significance of infection of both *F. milksi* and *F. hirthi* species seemed to be unimportant in healthy animals. However, one fatal case of *F. milksi* infection was described (Corwin et al., 1974). The same, intensive to fatal infections of *F. hirthi* species were encountered as the result of the immunosuppressive drug administration, of the hyperadrenocorticism, or of the immunity disorders in several cases (Craig et al., 1978; Geisel, 1979; August et al., 1980; Genta and Schad, 1984; Rubash, 1986; Valentine and Georgi, 1987; Pinckney et al., 1988; Carrasco et al., 1997; Torgerson et al., 1997). The significance of an opportunistic infection could not be neglected, either. In toxicologic studies, the diagnostics of the lung filaroidosis was very important because of possible misinterpretation of the histopathological findings (Hirth and Hottendorf, 1973; Bahnemann and Bauer, 1994). From this point of view, an efficient control of filaroidosis should be very important (Bauer and Bahnemann, 1996).

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HOW TO EVALUATE THE RESULTS OF RESEARCH: WHAT HAS NOT BEEN PUBLISHED HAS NOT BEEN DONE

Information and advice for postgraduate students and new research workers. Warning about the importance of publications, the selection of magazine, and the responsible preparation of manuscripts.

JAK HODNOTIT VÝSLEDKY VÝZKUMU: CO NEBYLO UVEŘEJNĚNO, NEBYLO UDĚLÁNO

Objektivní hodnocení výsledků výzkumu je předpokladem účelného vynakládání finančních prostředků a podpory perspektivních pracovníků. Nelze jej dosáhnout jinak, než důsledným uplatňováním zásady, že výsledky musí být uveřejněny v časopisech s náročným lektorským řízením. Posuzování náročnosti časopisů je samozřejmě předmětem častých diskusí. Jedním z nejvýznamnějších kritérií je impakt faktor časopisu, který vyjadřuje ohlas na práce v něm uveřejněné. Počet citací každé práce je odvozen především od její kvality a od jejího zaměření. Nicméně ani sebelepší výsledky, uveřejněné v jiné řeči než v angličtině a v časopisu, vycházejícím v malém nákladu a uveřejňujícím vedle dobrých i práce nevalného významu nebo práce zcela špatné, budou mít ohlas menší, než kdyby vyšly v mezinárodním časopisu v angličtině, byly posouzeny a k uveřejnění doporučeny lektory s velkými zkušenostmi, zcela nezávislými na autorech, na jejich pracovišti i na instituci, která na řešení poskytla finanční prostředky. Výsledky uveřejněné v časopisu sledovaném některým z rozšířených databázových systémů nebo referátových časopisů, se dostanou nejrychleji k lidem, řešícím podobnou problematiku a najdou tak svoje uplatnění. Výsledky, uveřejněné v dobrém časopisu, které se z nejrůznějších důvodů nestanou v nejbližší době předmětem zájmu a citací, se přesto zcela neztratí. Pokud jsou dobré, mohou najít svoje uplatnění později.

Uveřejnit výsledky v náročném časopisu vyžaduje od řešitelů mnoho znalostí i námahy. Především musí pracovat na nosném tématu. Každý nový poznatek je přínosem, ale ne každý problém je stejně aktuální a významný. To neznamená, že by měli pracovníci ve výzkumu přebíhat od jednoho tématu k druhému podle toho, co právě a někdy jen zdánlivě nebo dočasně „frčí". Nedosáhli by tak dalšího předpokladu úspěchu: neměli by v oboru dostatek znalostí, aby byli schopni vymezit správně téma výzkumu, vyslovit hypotézu, kterou považují za vhodné ověřit, a zvolit postup, kterým lze ověření této hypotézy dosáhnout. Pokud překonají tato úskalí, musí mít řešitelé také podmínky pro použití nejlepších postupů a pro provedení experimentů v rozsahu potřebném pro spolehlivé vyslovení závěru, že hypotéza platí nebo že byla jednoznačně vyvrácena. K tomu jsou nezbytné kromě zkušeností především peníze. Nesmí však chybět ani pracovitost a trpělivost hraničící často s obětováním se svému problému. Ve výzkumu lze jen výjimečně dosáhnout úspěchu s přístupem „zaměstnance". Úspěchu dosahují především ti, kteří mají nezdolnou touhu objevovat a jsou ochotni svojí zálibě věnovat čas neměřitelný pracovní dobou. Výsledky pozorování, studia nebo experimentu musí být pečlivě zaznamenány, kvantitativní údaje vyjádřeny graficky a vyhodnoceny vhodnými statistickými postupy a kriticky porovnány se známými údaji. Vše musí být srozumitelně sepsáno, mnohokrát přečteno, prodiskutováno se spoluautory i s dalšími kolegy, podrobeno jazykové kontrole a odesláno do nejvhodnějšího časopisu. Připomínky lektorů musí být přijímány s pokorou, což neznamená, že lektor má vždy pravdu a že nelze mít k jeho požadavkům výhrady. Vždy je však nutné jednoznačně uvést, které připomínky a jak byly přijaty a proč jiné autoři přijmout nemohou. Osud práce je nakonec v rukou autorů i v případě, že se setkají s lektory nekompetentními nebo zaujatými: dobré výsledky mohou vždy uplatnit v jiném, třeba i lépe hodnoceném časopisu. Dostat výsledky výzkumu do publikovaného článku však vyžaduje, aby byly skutečně kvalitní a řešitelé musí o jejich uveřejnění usilovat. Příprava publikace nezačíná až po ukončení řešení, což si myslí mnozí řešitelé grantů. Úspěch publikace se zakládá již při studiu literatury a přípravě projektu, při promýšlení hypotézy a metodiky jejího ověření, při zpracovávání výsledků do tabulek v pracovních protokolech a při ověřování alternativních možností jejich grafického zpracování, při volbě statistických metod a při interpretaci výsledků a jejich porovnání s názory již uveřejněnými. Skoro by se dalo říci, že kdo stačí tato doporučení plnit, nemá vlastně s přípravou rukopisu žádnou práci. Tak to samozřejmě není. Kdo ale takto nepostupuje, má malou šanci dosáhnout úspěchu uveřejněním svých výsledků co nejdříve po jejich dosažení a v co nejlepším časopisu.

Požadavek uveřejnění výsledků není šikanováním řešitelů grantů. Jeho splněním nepřinášeji nikomu žádnou obět, protože publikace je nejen nezbytným předpokladem doložení odpovědně využitých finančních prostředků, ale i osobním zájmem a povinností každého, kdo se výzkumem profesionálně zabývá. Je také nezbytným předpokladem pro získání finančních prostředků pro další výzkum i pro dosažení osobního uznání udělením vyšších vědeckých a pedagogických hodností a zařazením do vyšších platových tříd.

Požadavek uveřejnění výsledků platí pro téměř všechny oblasti výzkumu. Výjimkou je snad výzkum obranný nebo komerčně využitelný, pokud by předčasným uveřejněním výsledků byly ohroženy zájmy toho, kdo práci objednal a financoval. Grantové agentury, podporující základní výzkum a přidělující granty v soutěži podaných návrhů, mají naopak velký zájem na uveřejňování výsledků, protože to je jediný způsob ověření, jak byly naplněny záměry řešitelů, jejichž splnění agentura udělením grantu umožnila. Jiný způsob, např. předkládání podrobných zpráv a jejich posuzování oponenty je nejen zdlouhavé, ale může být i méně objektivní. Ukončování všech projektů uveřejněním dosažených výsledků je proto v zájmu samotných řešitelů projektů.

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THE JOURNAL VETERINÁRNÍ MEDICÍNA ENTERS THE YEAR 2000 WITH ITS 45th VOLUME

ČASOPIS VETERINÁRNÍ MEDICÍNA VSTUPUJE SVÝM 45. ROČNÍKEM DO ROKU 2000

The journal Veterinární medicína (Vet. Med. – Czech) enters the year 2000 offering a new web page layout which is part of the information network of the Veterinary Research Institute in Brno <http://www.vri.cz>. From the main menu, select Vet Med-Czech, where you will be able to search by all authors' names or key words for all volumes since 37, 1992. After the authors' names, title of article and quotations have been displayed, it is possible to ask for abstracts of all articles selected or only articles highlighted. After acquainting oneself with the abstracts, it is possible to highlight the articles of which the web page visitor wishes copies to be sent by mail. The request is sent by electronic mail and dealt with immediately.

The remaining information on the main page of the journal "Veterinary Medicine" are the contents of the last few issues and the issues currently in print. Separate offprints of these articles may also be requested directly from the web page.

The web page also offers instructions for authors in a Czech or English version and the possibility of sending e-mail to the editorial board.

I am confident that the possibility of easily searching for published articles and acquiring them in full will contribute to the wider exploitation of results and to greater citation of the journal and the authors who publish in it. Authors may significantly influence the frequency of citation of their work and the impact factor of the magazine by sending separates to their colleagues who are working in the same area and to the authors of articles which were cited in their work published in Veterinary Medicine. The most important presumption for increasing the impact factor, which is an expression for the citation frequency of each journal, is the high quality of published contributions. This is above all in the hands of authors, and is checked by a peer review system. In the last issue of the preceding volume you will have undoubtedly noticed the numbers of foreign readers whom the editorial board thanked for their cooperation and who significantly contribute to the moderate increase in the impact factor of our magazine, which was 0.231 in 1998.

I would like to thank all authors, referees, members of the editorial board and the editor-in-chief Ing. Z. Radošová for their help and wish them health and much success in their work and personal lives in the coming year.

Prof. MVDr. Karel Hruška, CSc.
Chairman of the Editorial Board

Časopis Veterinární medicína vstupuje do roku 2000 s nabídkou nového uspořádání web stránky <http://www.vri.cz>, která je součástí informací Výzkumného ústavu veterinárního lékařství v Brně. Z hlavního menu se otevře Vet Med-Czech, kde najdete vyhledávací program podle všech autorů nebo podle klíčových slov od roku 1992. Po zobrazení autorů, názvu článku a citace je možné vyžádat abstrakty všech vybraných článků nebo jen článků, zvolených zaškrtnutím. Po seznámení s abstrakty je dále možno označit články, jejichž kopii si návštěvník této web stránky přeje zaslat poštou. Požadavek je elektronickou poštou doručen a okamžitě vyřízen.

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Volume 45, No. 1, January 2000

CONTENTS

Tančin V., Kraetzl W. D., Schams D., Mihina Š., HeténYi L.: The oxytocin secretion and milk letdown during milking immediately after the change of milking and housing conditions (in English) ... 1

Antalíková L., Koubková M., Rozinek J., Jílek F.: Immunocytochemistry of heat shock proteins HSP70 in pig liver after a parasitic invasion (in English) ... 5

Rodák L., Šmíd B., Valíček L., Zajac L.: Demonstration by ELISA of antibodies to Aujeszky disease virus in porcine blood serum and meat juice (in English) ... 13

Nečas A.: Rate of neurologic recovery as an indicator of long-term prognosis in dogs with surgically treated thoracolumbar disc disease (in English) ... 19

Vajner L., Vortel V., Brejcha A.: Lung filaroidosis in the Beagle dog breeding colony (in English) 25

INFORMATION

Hruška K.: How to evaluate the results of research: what has not been published has not been done ... 31

Hruška K.: The journal Veterinární medicína enters the year 2000 with its 45th volume ... 32

VETERINÁRNÍ MEDICÍNA

Ročník 45, č. 1, Leden 2000

OBSAH

Tančin V., Kraetzl W. D., Schams D., Mihina Š., HeténYi L.: Sekrécia oxytocínu a ejekcia mlieka počas dojenia bezprostredne po zmene podmienok dojenia a ustajnenia ... 1

Antalíková L., Koubková M., Rozinek J., Jílek F.: Imunocytochemická detekce stresových proteinů HSP70 v játrech prasat po parazitární invazi ... 5

Rodák L., Šmíd B., Valíček L., Zajac L.: Průkaz protilátek proti viru Aujeszkyho choroby v krevním séru a masové tekutině prasat ELISA metodou ... 13

Nečas A.: Rychlost obnovy neurologických funkcí jako ukazatel dlouhodobé prognózy u psů s chirurgicky léčeným onemocněním torakolumbálních meziobratlových plotének ... 19

Vajner L., Vortel V., Brejcha A.: Plicní filaroidosis v chovné kolonii psů plemene beagle ... 25

INFORMACE

Hruška K.: Jak hodnotit výsledky výzkumu: co nebylo uveřejněno, nebylo uděláno ... 31

Hruška K.: Časopis Veterinární medicína vstupuje svým 45. ročníkem do roku 2000 ... 32

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